PHENYLALANINE BIOSYNTHESIS IN *Pseudomonas aeruginosa* AND *Xanthomonas campestris*

By

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by

Wei Gu

This dissertation is dedicated to my Mom and Dad, who gave me an opportunity to be in this world, to experience the enjoyment and stress which has accompanied the fulfillment of this dissertation effort.

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Abstract of Dissertation Presented to the Graduate School
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PHENYLALANINE BIOSYNTHESIS IN *Pseudomonas aeruginosa* AND *Xanthomonas campestris*

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A periplasmic route of *L*-phenylalanine biosynthesis was demonstrated in *Pseudomonas aeruginosa*. Component enzymes include monofunctional chorismate mutase (denoted $\mathrm{AroQ_f}$), cyclohexadienyl dehydratase (denoted PheC), and aromatic aminotransferase. SDS-PAGE analysis of partially purified $\mathrm{AroQ_f}$ indicated a subunit molecular mass of 22 kDa. Results from gel filtration showed native $\mathrm{AroQ_f}$ to be a 22-kDa monomer. *pheC* was inactivated by gene insertion, thus providing a tool for full elucidation of its function. Periplasmic aromatic aminotransferase was resolved into multiple species after hydroxylapatite chromatography, and one of them (a homodimer having 34-kda subunits) was partially purified. Whether an already available clone might possibly be synonymous with aromatic aminotransferase was evaluated. PhhC was shown to be capable of *in vivo* function as both an aromatic and an aspartate aminotransferase. PhhC has been purified to homogeneity from *E. coli*. It has a monomeric mass of 43,276 Da. PhhC showed a heterogenous elution profile following hydroxylapatite chromatography, and it appears to be an almost equal mixture of charge isoforms at equilibrium. PhhC is a catabolic enzyme, and it appears not to be a periplasmic protein.

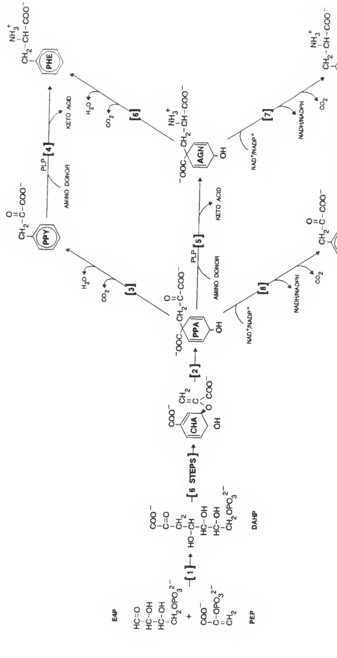
The gene (denoted *aroQ*$_p$*-pheA*) encoding the bifunctional P-protein (chorismate mutase-P/prephenate dehydratase) from *Xanthomonas campestris* was sequenced. It exhibited 51% amino-acid identity with the *Pseudomonas stutzeri* homolog. It also possesses a unique 40-residue amino-terminal extension which is lysine-rich. A molecular mass of 81 kDa was estimated for the native protein, indicating that the active enzyme species is a homodimer. When AroQ$_p$-pheA was overexpressed by a T7 translation vector, unusual inclusion bodies were formed which exhibit a sphagetti-like macromolecular configuration. Insoluble protein collected at low-speed centrifugation was fully active, and the single band obtained via SDS-PAGE was used for N-terminal amino-acid sequencing. A perspective on the evolutionary relationships of AroQ$_f$ and PheA proteins and the AroQ$_p$-PheA family of proteins is presented.

CHAPTER 1
LITERATURE REVIEW

The Aromatic Amino Acid Pathway

The biosynthesis of all three aromatic amino acids begins with a common trunk of intermediates initiated with the condensation of erythrose-4-phosphate and phosphoenol pyruvate by 3-deoxy-**D**-*arabino*-heptulosonate-7-phosphate (DAHP) synthase and ending with chorismate, the last intermediate common to all three aromatic amino acids (Fig. 1-1). From chorismate three major branches lead to *L*-phenylalanine, *L*-tyrosine, and *L*-tryptophan. The phenylpyruvate route for conversion of chorismate to *L*-phenylalanine and the 4-hydroxyphenylpyruvate route for conversion of chorismate to *L*-tyrosine were established by classic studies in enteric prokaryotes such as *Escherichia coli* (Cotton and Gibson, 1965). In 1974, an alternative pathway of *L*-tyrosine biosynthesis was described in species of cyanobacteria in which prephenate is transaminated to *L*-arogenate (then designated as pretyrosine), and *L*-arogenate was then converted to *L*-tyrosine by arogenate dehydrogenase (Stenmark *et al.*, 1974). Although not present

Fig. 1-1. Biochemical pathway for biosynthesis of aromatic amino acids. Erythrose-4-phosphate and phosphoenolpyruvate are first condensed to form 3-deoxy-**D**-*arabino*-heptulosonate 7-phosphate (DAHP) via the action of DAHP synthase (1). Chorismate is the last intermediate common to all three aromatic amino acids. The post-prephenate pathways to *L*-phenylalanine and *L*-tyrosine, where considerable biochemical diversity formed in nature, are shown as a composite scheme. Abbreviations: E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-**D**-*arabino*-heptulosonate 7-phosphate; CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; PHE, *L*-phenylalanine; ANG, *L*-arogenate; HPP, 4-hydroxyphenylpyruvate; TYR, *L*-tyrosine; PLP, pyridoxal-5'-phosphate. Enzymes are numbered as follows: [1] DAHP synthase; [2] chorismate mutase, encoded by $aroQ_f$, or $aroQ_r$, or $aroQ_p$, or $aroQ_t$, or $aroQ_d$; [3] prephenate dehydratase, encoded by *pheA* or *'pheA*; [4] aromatic aminotransferase; [5] prephenate aminotransferase; [6] arogenate dehydratase; [7] arogenate dehydrogenase; [8] prephenate dehydrogenase, encoded by *tyrA* or *'tyrA*; [9] aromatic aminotransferase. If a single dehydratase catalyzes both reactions 3 and 6, it is denoted cyclohexadienyl dehydratase; if a single dehydrogenase catalyzes both reactions 7 and 8, it is denoted cyclohexadienyl dehydrogenase. The bifunctional P-protein catalyzes the sequential reactions of chorismate mutase and prephenate dehydratase. The bifunctional T-protein catalyzes the sequential reactions of chorismate mutase and prephenate dehydrogenase. See "Rationale for Terminology" for definition details of $aroQ_f$, $aroQ_r$, $aroQ_p$, $aroQ_t$, $aroQ_d$, *pheA*, *'pheA*, *tyrA* and *'tyrA*.


E4P
PEP
[1]
DAHP
[6 STEPS]
CHA
[2]
PPA
[3]
H2O
CO2
PPY
PLP
[4]
AMINO DONOR
KETO ACID
PHE
[5]
AGN
[6]
[8]
NAD+/NADP+
NADH/NADPH
HPP
[9]
[7]
TYR

in cyanobacteria, an *L*-arogenate-mediated sequence to *L*-phenylalanine was also found to exist in some microorganisms (Byng *et al.*, 1982) and in higher plants (Jensen, 1986).

The Complexity of the Phenylalanine Pathway in Microorganisms

The biochemical pathway utilized for the biosynthesis of aromatic amino acids is strikingly diverse in microorganisms (Jensen, 1992). *P. aeruginosa* and *E. coli* are landmark organisms which differ from one another and for which a great deal of information exists about this pathway. Organisms such as *P. aeruginosa* typify a large group of gram-negative bacteria which differ from *E. coli* in that they maintain two separate pathways to *L*-phenylalanine. The nature of these dual pathways to *L*-phenylalanine is the focal point of this dissertation.

E. coli possesses a bifunctional protein, chorismate mutase-P/ prephenate dehydratase (P-protein) encoded by $aroQ_p$-*pheA*. The P-protein from *E. coli* has been purified and characterized (Davidson et al., 1972). The enzyme is a homodimer with an apparent subunit molecular weight of 40,000 (Davidson *et al.*, 1972). Both activities are feedback inhibited by *L*-phenylalanine (Dopheide *et al.*, 1972). Evidence from chemical modification (Gething and Davidson, 1977a; and 1977b), kinetic studies (Dopheide *et al.*, 1972; and Duggleby *et al.*, 1978), and the isolation of mutants which were defective in prephenate dehydratase (PheA), or chorismate mutase-P (AroQ$_p$), or both activities (Baldwin and Davidson, 1981), strongly suggest that the P-protein has separate catalytic sites for chorismate mutase and prephenate dehydratase. Native P-protein tends to form tetramers at high protein concentration or in the presence of *L*-phenylalanine (Davidson *et al.*, 1972). The synthesis of P-protein is derepressed when cells are starved for *L*-phenylalanine (Brown and Somerville, 1971). From classic genetic studies of various mutants which were derepressed for synthesis of *aroQ*$_p$-*pheA* gene product (Im and Pittard, 1973; Gowrishankar and Pittard, 1982), and from more recent molecular-genetic analyses of the upstream leader region (Zurawski et al., 1978), it is now known that the *aroQ*$_p$-*pheA* expression is regulated solely by attenuation at a transcription terminator located upstream of *aroQ*$_p$-*pheA*.

The last reaction in the phenylalanine pathway involves transamination of phenylpyruvate, using *L*-glutamate as the amino donor. Assisted by the isolation of mutants lacking individual aminotransferase enzymes (Gelfand and Steinberg, 1977) coupled with the development of methods for the separation of aminotransferase activities (Powell and

Morrison, 1978), we now know that three enzymes need to be considered *in vivo*: the aromatic aminotransferase encoded by *tyrB*; the aspartate aminotransferase encoded by *aspC*; and the branched-chain amino acid aminotransferase encoded by *ilvE*. Only mutants lacking all three activities require both phenylalanine and tyrosine (Gelfand and Steinberg, 1977). The nucleotide sequences of *tyrB*, *aspC*, and *ilvE* have been determined (Fotheringham *et al*., 1986; and Kuramitsu *et al*., 1985). AspC and TyrB are homologous proteins and the members of the same subfamily (I_α) of Family I within the aminotransferase superfamily, while IlvE lacks homology to either AspC or TyrB, and is a member of the Family III within the aminotransferase superfamily (Jensen and Gu, 1996).

Superfamily B is a lineage of gram-negative bacteria in which the evolutionary history of aromatic biosynthesis has been studied in much more detail than in any other phylogenetic grouping (Jensen, 1985). Within this superfamily, organisms possessing a single pathway or dual pathways for phenylalanine biosynthesis are shown (Fig. 1-2). Dual enzymatic routes to *L*-phenylalanine was first described in *P. aeruginosa* (Patel *et al*., 1977). Like *E. coli*, *P. aeruginosa* possesses a bifunctional P-protein (Patel *et al*., 1977). Both activities of the *P. aeruginosa* P-protein are not only feedback inhibited by *L*-phenylalanine, but prephenate

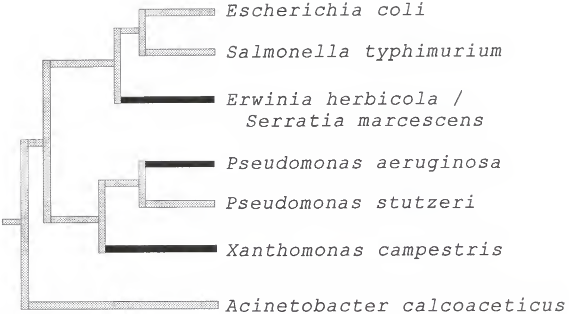


Fig. 1-2. Some representative organisms in superfamily B which possesses a single pathway (dotted bar) or dual biosynthetic pathways (solid bar) to *L*-phenylalanine. Approximate phylogenetic relationships of the organisms specified are shown on the dendrogram.

dehydratase is activated by *L*-tyrosine (Patel *et al.*, 1977). In addition to the bifunctional P-protein, an unregulated route to *L*-phenylalanine is also present, which consists of monofunctional chorismate mutase-F (AroQ$_f$), aromatic aminotransferase, and cyclohexadienyl dehydratase.

P. aeruginosa pheC has been cloned and sequenced, and the cyclohexadienyl dehydratase gene product has been purified (Zhao *et al.*, 1992). The enzyme utilizes prephenate and arogenate as alternative substrates. The enzyme activity is not subject to allosteric control by phenylalanine, tyrosine, and tryptophan when present singly or in combination (Zhao *et al.*, 1992). Amino acid sequence comparison does not show obvious homology with the dehydratase domains of known P-proteins, but does reveal a conserved motif which includes an essential threonine residue at the catalytic site (Zhao *et al.*, 1992). A monofunctional chorismate mutase-F (AroQ$_f$) was found in *P. aeruginosa* which could be separated from P-protein by ion-exchange chromatography (Patel *et al.*, 1977). It has a molecular weight of 27,000 , and the activity is not inhibited by *L*-phenylalanine, *L*-tyrosine, or prephenate. *P. aeruginosa* possesses five species of aminotransferase capable of function as aromatic aminotransferase *in vitro* (Whitaker *et al.*, 1982). Mutants lacking one species and two species of the five aminotransferase have phenotypes of *L*-phenylalanine bradytrophy and auxotrophy, respectively. Major *in vivo* roles have been postulated.

Xanthomonas campestris, a representative of the Group V pseudomonads, possesses a bifunctional P-protein bearing the activities of both chorismate mutase and prephenate dehydratase (Whitaker *et al.*, 1985). The prephenate dehydratase was slightly stimulated by *L*-tyrosine, both prephenate dehydratase and chorismate mutase activities were feedback inhibited by *L*-phenylalanine when substrate concentrations were fixed at about the K_m level. In addition to this route, an unregulated cyclohexadienyl dehydratase and chorismate mutase-F ($AroQ_f$) similar to those of *P. aeruginosa* were found to exist in this organism.

The Periplasmic Phenylalanine Biosynthetic Pathway in Gram-negative Organisms

Periplasm or periplasmic space was defined as a cellular compartment lying between the outer membrane and the cytoplasmic membrane of gram-negative bacterial cell (Beacham, 1979). A majority of the enzymes localized within the periplasm of bacterial cells was found to be hydrolytic (degradative) enzymes, or binding proteins which are involved in active transport (Beacham, 1979).

Zhao *et al.* (1993a) reported the DNA sequence of the *pheC* gene from *P. aeruginosa*. He found that there was a 25-residue region at the N-terminus of PheC cleaved from the N-terminus of a preprotein formed in *E. coli*. The amino acid composition of the 25-residue peptide was typical of signal sequence for periplasmic proteins. The cyclohexadienyl dehydratase was indeed released from *P. aeruginosa* and *E. coli* cultures carrying the *pheC* gene when techniques employing spheroplast formation, osmotic shock or chloroform treatment were used. Electron microscopy using immunogold labelling showed an apparent localization of cyclohexadienyl dehydratase at the polar regions of the periplasmic space in *E. coli*. This was the first enzyme involving phenylalanine biosynthesis to be localized in the periplasmic space. Later on, a monofunctional chorismate mutase (AroQ$_f$) from *Erwinia herbicola* was also found to be located in the periplasm (Xia *et al*., 1993b).

pheC and *aroQ*$_f$ are widely distributed among gram-negative prokaryotes (Ahmad and Jensen, 1988), but physiological roles of their encoding proteins are unclear. Using *P. aeruginosa* and *X. campestris* as representative species of important divergent lineages which both exhibit dual pathways to *L*-phenylalanine, this dissertation is an initial effort to characterize the physiological, enzymological and molecular-genetic nature of the co-existing cytoplasmic and periplasmic pathways to *L*-phenylalanine that are widespread in gram-negative bacteria.

Rationale for Terminology

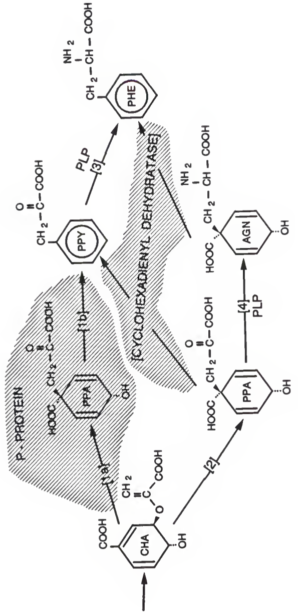
It is unfortunate that the early *E. coli* auxotrophs for phenylalanine and tyrosine were denoted *pheA* and *tyrA* before it was known that each gene product was bifunctional, carrying two domains. Each catalytic domain corresponds to individual proteins in many other organisms. It is becoming increasingly confusing, especially where multiple molecular-genetic comparisons are being made, to use acronyms whose meaning is different in different organisms. We therefore propose the following definitions (see Fig. 1-1 for the locations of these corresponding enzymes in the pathway). *pheA* encodes monofunctional prephenate dehydratase; '*pheA* encodes the prephenate dehydratase domain of a P-protein. *tyrA* encodes monofunctional prephenate dehydrogenase; '*tyrA* encodes the prephenate dehydrogenase domain of a T-protein. Monofunctional, allosterically-insensitive chorismate mutase (commonly called chorismate mutase-F) is denoted $aroQ_f$. Monofunctional, allosterically-sensitive chorismate mutase (chorismate mutase-R) is denoted $aroQ_r$. The chorismate mutase domain of the bifunctional P-protein is denoted $aroQ_p$, whereas the chorismate mutase domain of the bifunctional T-protein is denoted $aroQ_t$. The chorismate mutase domain co-existing with a DAHP synthase domain on a bifunctional protein is denoted *aroQ*$_d$. The P-protein is called AroQ$_p$-PheA and the T-protein is called AroQ$_t$-TyrA.

CHAPTER 2
THE SEPARATELY COMPARTMENTED PHENYLALANINE PATHWAYS OF *PSEUDOMONAS AERUGINOSA*

Introduction

P. aeruginosa has been known for some time to possess a second pathway of phenylalanine biosynthesis (Fig. 2-1) (Patel *et al.*, 1977; and Fiske *et al.*,1983). An *aroQ*$_p$-*pheA* mutant of *P. aeruginosa* was shown to have a phenotype of phenylalanine bradytrophy due to the presence of AroQ$_f$ (encoded by *aroQ*$_f$), the *pheC*-encoded cyclohexadienyl dehydratase (PheC) and one or more unspecified aromatic aminotransferases (Berry *et al.*, 1987). The gene encoding PheC has been cloned and sequenced (Zhao *et al.*, 1992). Unexpectedly, PheC was found to be a periplasmic protein (Zhao *et al.*, 1993a). Therefore, one might expect AroQ$_f$ and aromatic aminotransferase activity to be located in the periplasm as well. My preliminary data showed that in addition to PheC, AroQ$_f$ and aromatic aminotransferase were indeed detectable in the periplasmic fraction of *P. aeruginosa* wild type PAO1. The localization of biosynthetic enzymes in the periplasm is unprecedented. The leaky auxotrophy of the aforementioned *aroQ*$_p$-*pheA* mutant shows

Fig. 2-1. Dual biosynthetic route to *L*-phenylalanine in *Pseudomonas aeruginosa*. The bifunctional P-protein (denoted by shading) consists of chorismate mutase [1a] and prephenate dehydratase [1b] domains. The broad-specificity cyclohexadienyl dehydratase catalyzes the two reactions indicated by shading: prephenate dehydratase (left) or arogenate dehydratase (right). Enzyme [2] is the previously denoted monofunctional chorismate mutase-F (herein denoted $\mathrm{AroQ_f}$). Reactions [3] and [4] refer to a multiplicity of aminotransferase enzymes with overlapping specificities which transaminate phenylpyruvate and prephenate, respectively. Abbreviations: CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; PHE, *L*-phenylalanine; AGN, *L*-arogenate; PLP, pyridoxal-5'-phosphate.


P - PROTEIN
[CYCLOHEXADIENYL DEHYDRATASE]
CHA
PPA
PPY
PHE
AGN
[1a]
[1b]
[2]
[3]
[4]
PLP
PLP
COOH
OH
CH2
C
O
HOOC
CH2—C—COOH
NH2
CH2—CH—COOH

that the periplasmic pathway does not ordinarily provide sufficient phenylalanine for cells to sustain the normal growth rate. Thus, PheC, AroQ_f and periplasmic aromatic aminotransferase may have some unknown functional role beyond their capacity to be the components of a backup pathway to phenylalanine. In this chapter, I will present evidence for the presence of the three enzymes needed to convert chorismate to *L*-phenylalanine in the periplasmic space of *P. aeruginosa*, describe the purification of two of the enzymes (AroQ_f and aromatic aminotransferase), demonstrate the isolation of a *pheC* mutant by *in vivo* gene inactivation, and discuss the possible role of this periplasmic pathway.

Materials and Methods

Bacterial Strains, Plasmids, and Media

All bacterial strains and plasmids used in this study are listed in Table 2-1.

Luria-Bertani (LB) medium was used as enriched medium (Silhavy *et al.*, 1984). The minimal medium used was the M-9 formulation, except for *E. coli* DL39 which was grown in the medium of LeMaster and Richards (1988). Where indicated, ampicillin was added to media at 100 µg/ml, kanamycin was added at 35 µg/ml, thiamine was added at 17 µg/ml, and $HgCl_2$

Table 2-1. Bacterial strains and plasmids.

Strain/ plasmid	Relevant characteristics	Source and reference
E.coli		
DH5α	F$^-$, ϕ80d*lacZ*Δ*M15*, *recA1*, *endA1*, *gyrA96*, *supE44*, *hsdR17*(r_K^-, m_K^+), Δ(*lacZYA-argF*)*U169*, *thi-1*, *relA1*, *deoR*	Bethesda Research Laboratories
JP2255	*aroF363*, *pheA361*, *pheO325*, *tyrA382*, *thi-1*, *strR712*, *lacY1*, *xyl*-15	Baldwin and Davidson, 1981
JP2255-T	JP2255 harbouring plasmid pJG-T	This study
S17-1	[RP4-2 (Tc::Mu)(Km::Tn7) Tra (IncP)], *pro*, *hsd*R, *rec*A, *thi*-1 Tp^r, Sm^r	Simon *et al.*, 1983
DL39	λ^- *aspC13 fnr-25 ilvE12 tyrB507*	LeMaster and Richards, 1988
B171	Wild-type EPEC 0111:NM; carries the 80-kb EAF plasmid and the 90-kb Tet^r-Str^r plasmid	Girón *et al.*, 1993
B171-5	B171 lacking the EAF plasmid; retains the 90-kb Tet^r-Str^r plasmid	as above
B171-8	B171 lacking the 90-kb Tet^r-Str^r plasmid; retains the EAF plasmid	as above
0127:H6	Wild-type EPEC 0127:H6	as above
P.aeruginosa		
PAO1	prototroph	Holloway, 1969
PAT1051	*pheA*	Waltho, 1972
JG007	PAO1 derivative (Hg^r, *phe*C)	This study
PAO381	Non-mucoid	Matin *et al.*, 1993
PAO671	*mucB*::T_c^R, Mucoid	as above
PG201/rhlR$^-$	rhamnolipid-deficient, elastase-negative, carbenicillin^R	Ochsner and Reiser, 1995

Table 2-1 (continued)

Strain/ plasmid	Relevant characteristics	Source and reference
PG201/rhlI$^-$	rhamnolipid-deficient, elastase-negative, carbenicillin^R	as above
NP-72	phenylalanine bradytroph	Whitaker *et al.*, 1982
RW8-24	phenylalanine auxotroph	as above
JS104	PAO1 derivative (Hg^r, *phh*C)	J. Song
Plasmid		
pUC18	Ap^r, *lacI'POZ'*	Yanisch-Perron *et al.*, 1985
pGEM-5zf(+)	Ap^r, *lacI'POZ'*	Promega
pACYC177	Ap^r, Km^r	Chang and Cohen, 1978
pJZ5f	2.5-kb *Nco*I-*Nco*I fragment of *tyrC* subcloned into pGEM-5Zf(+) at *Nco*I site	Zhao *et al.*, 1993
pJG-T	2.5-kb *Nco*I-*Nco*I fragment of pJZ5f cloned into pACYC177 at *Hinc*II site	This study
pUFR004	ColE1,Cm^r, Mob$^+$, *mob*(P)	Defeyter *et al.*, 1990
pJZ1g	1.1-kb *Sph*I-*Sma*I fragment of *pheC*	Zhao *et al.*, 1992
pJG-C	0.6-kb *Bal*I-*Stu*I fragment from pJZ1g subcloned into pUFR004 at *Sma*I site	This study
pDE-Hg	Ap^r, Hg^r	Esser *et al.*, 1990
pZ1918	Ap^r	Schweizer, 1993
pJG-Hg	Replacement of *lacZ* in pZ1918 with the 5.2-kb *mer* operon	This study

Table 2-1 (continued)

Strain/ plasmid	Relevant characteristics	Source and reference
pJG-C_{Hg}	5.2-kb *mer* operon inserted into pJG-C at *Hind*III site	This study
pJS3A	3.7-kb *Bam*HI-*Hind*III fragment of *phhB-phhC* subcloned into the *Bam*HI-*Hind*III site of pUC18	J.Song
pJS3B	3.7-kb *Bam*HI-*Hind*III fragment of *phhB-phhC* subcloned into the *Bam*HI-*Hind*III site of pUC19	J.Song

was added at 15 µg/ml. Medium was solidified with 2% (wt/vol) agar.

Extract Preparation and Enzyme Assays

E. coli transformants or *P. aeruginosa* cells were grown at 37°C with vigorous shaking in LB or minimal medium supplemented with appropriate antibiotics. Cells were harvested in the late-exponential phase of growth by centrifugation and resuspended in 50 m*M* potassium phosphate buffer (pH 7.0), containing 1 m*M* DTT. The cells were disrupted by sonication using an Ultratip Labsonic System (Lab-Line Instruments, Inc. Melrose Park, IL), and then centrifuged at 150,000 x *g* for 65 min at 4°C. The supernatant fraction was collected and passed through a 1.5 x 5.5 cm DG-10 Sephadex column (Bio-Rad). The resulting desalted preparation is designated as crude extract.

Chorismate mutase and prephenate dehydratase were assayed spectrophotometrically by the method of Cotton and Gibson (1965) as modified by Ahmad and Jensen (1988), using 1 m*M* chorismate and 1 m*M* prephenate, respectively. Arogenate dehydratase was assayed by measuring the formation of *L*-phenylalanine using HPLC (Fischer and Jensen, 1987). Aromatic aminotransferase was assayed as described before (Gu *et al.*, 1995). Phenylalanine and α-ketoglutarate were used as the substrates, unless indicated otherwise. Shikimate dehydrogenase was assayed by monitoring the reduction of NADP$^+$ at 340 nm (Chaudhuri *et al.*, 1987).

Separation of Periplasmic and Cytoplasmic Fractions of P. aeruginosa

A modified magnesium shock procedure (Kessler and Safrin, 1994) was used to fractionate cytoplasmic and periplasmic enzymes in *P. aeruginosa*. *P. aeruginosa* strains were grown to late-exponential phase in 300 ml of M-9 salts/glucose medium. The culture was divided into two 150-ml parts and harvested by centrifugation at 6,000 x *g* at 4°C for 20 min. One part was resuspended in 5 ml of 50 m*M* potassium phosphate buffer (pH 7.0), and broken by sonication to make crude extract. The other part was resuspended in 20 ml of concentrated magnesium solution (0.2 *M* $MgCl_2$; 0.01 *M* Tris·HCl, pH 8.4; lysozyme 5 mg/ml), incubated at 25°C for 20 min, then centrifuged at 6,000 x *g* at 4°C for 20 min. The supernatant was saved as the wash fraction. The pellet was resuspended in 20 ml of cold shock solution (0.01 *M* $MgCl_2$; 0.01 *M* Tris·HCl, pH 8.4), incubated on ice for 20 min, then centrifuged at 6,000 x *g* at 4°C for 20 min. The resulting supernatant combined with the wash fraction was dialyzed against 50 m*M* (pH 7.0) potassium phosphate buffer overnight (use at least ten times volumn of the buffer, with one change of the buffer after about 5-6 hours), concentrated on a YM-10 membrane apparatus to 4 ml, and denoted as the periplasmic fraction. The remaining pellet was resuspended in 3 ml of 50 m*M* phosphate buffer (pH 7.0) and sonicated to yield the cytoplasmic fraction.

PheC activity was the marker protein for the periplasmic fraction, and shikimate dehydrogenase activity was the marker protein for the cytoplasmic fraction.

Partial Purification of AroQ$_f$ and a Periplasmic Aromatic Aminotransferase

A 20-liter culture of *P. aeruginosa* strain PAO1 (1-liter per 2.8-liter flask) was grown to late-exponential phase in M-9 salts/glucose medium, and the periplasmic fraction was separated from the rest of the cells as described above, but with a scaled-up protocol. All the buffers contained 1 m*M* DTT.

A 2.5 x 16 cm Bio-Gel HTP column previously equilibrated with 5 m*M* phosphate buffer (pH 7.0) containing 1 m*M* DTT was loaded with a 240-mg amount of protein, washed with 500 ml of starting buffer, and bound proteins eluted with 800 ml of phosphate buffer at pH 7.0 (potassium phosphate gradient from 5-400 m*M*). Fractions of 3.5 ml were collected, and chorismate mutase and aromatic aminotransferase activities were assayed.

AroQ$_f$. Fractions showing high chorismate mutase activity were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialyzed overnight against 50 m*M* phosphate buffer (pH 7.0) containing 1 m*M* DTT. An FPLC-connected Mono Q HR 5/5 anion-exchange column (Pharmacia Biotech) equilibrated with 50 m*M* phosphate buffer (pH 7.0) containing 1 m*M* DTT was loaded with the concentrated AroQ$_f$ fraction. The column was washed with 10 ml of the starting buffer and bound proteins were gradient-eluted with 20 ml of phosphate buffer (KCl gradient between 0 m*M* to 1 *M*). Fractions of 0.5 ml were collected, and those showing high chorismate mutase activity were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialyzed against 50 m*M* Tris-HCl buffer (pH 8.4) containing 1 m*M* DTT overnight, loaded onto the same Mono Q column which had been equilibrated with the same Tris-HCl buffer. The same elution regimen was used, except that the KCl gradient was from 0 m*M* to 350 m*M*. Fractions showing high chorismate mutase activity were pooled and dialyzed overnight against the above phosphate buffer containing 1 m*M* DTT and 150 m*M* NaCl. The fractions were concentrated by an Ultrafree®-15 centrifugal filter device (Millipore Corporation, Bedford, MA), and loaded onto an FPLC-connected Superdex®75 HR 10/30 (Pharmacia Biotech) column which had been equilibrated with 50 m*M* phosphate buffer (pH 7.0) containing 1 m*M* DTT and 150 m*M* NaCl. The same buffer was used for protein elution and fractions of 0.5 ml were collected. The fractions showing high chorismate mutase activity were pooled and concentrated by an Ultrafree®-15 centrifugal filter device (Millipore Corporation, Bedford, MA).

Periplasmic aromatic aminotransferase. Two peaks with aromatic aminotransferase activity eluted from the aforementioned hydroxylapatite column. When the major fractions with highest levels of activity was pooled and subjected to Mono Q chromatography as described above, two peaks of activity were resolved, one in the wash fraction (minor) and one in the gradient fraction (major). The second peak of aromatic aminotransferase (AAT-2g) was pooled and subjected to Superdex gel filtration as described above. The fractions showing high aromatic aminotransferase activity were pooled and concentrated by an Ultrafree®-15 centrifugal filter device (Millipore Corporation, Bedford, MA).

Purification of the Cloned Aromatic Aminotransferase (PhhC) from *E. coli* DL39

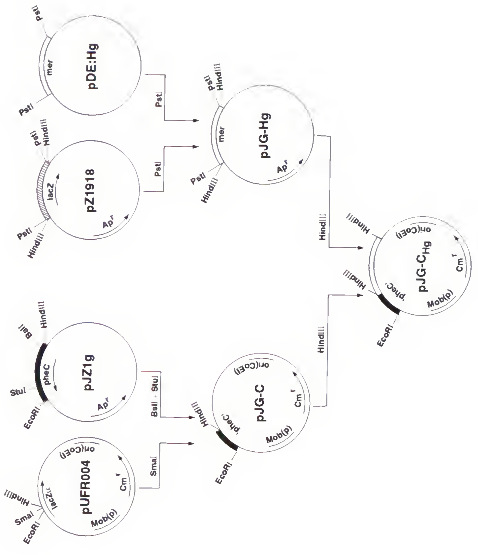
E. coli DL39 carrying plasmid pJS3A was grown at 37°C in 4 liters of LB medium supplemented with ampicillin in a gyratory shaker up to the late-exponential phase of the growth. The culture was harvested by centrifugation, and the pellet was resuspended in 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* DTT. The cells were disrupted by sonication and centrifuged at 150,000 x *g* for 65 min at 4°C. The crude extract was dialyzed against 2000 ml of the above buffer overnight with one change of the same buffer. This dialyzed extract (1184 mg of protein) was applied to a 3 x 50 cm DE-52 column equilibrated with the buffer. The column was washed with 800 ml of buffer, and bound protein was then eluted with 800 ml of a linear salt gradient (0-0.4 *M* KCl) prepared in the same buffer. Fractions (6 ml) were collected, assayed for aromatic aminotransferase activity. Fractions showing high enzyme activity were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialyzed overnight against 5 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* DTT. A 288-mg amount of protein was then loaded onto the bed of a 3 x 16 cm Bio-Gel HTP column previously equilibrated with the above buffer. The column was washed with 500 ml of starting buffer and protein was eluted by application of a potassium phosphate gradient (5 m*M* to 350 m*M*) in the same starting buffer. Fractions (3.5 ml) were collected, aromatic aminotransferase activity was assayed, and fractions showing high enzyme activity in the wash eluate were pooled and concentrated by means of an Amicon YM-10 membrane. The concentrated preparation was dialyzed overnight against 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* DTT and 0.15 *M* NaCl.

An FPLC-connected Superdex®75 HR 10/30 (Pharmacia Biotech) which had been previously equilibrated with the above buffer was used for a gel-filtration step. The protein was eluted with the same buffer. Fractions (0.5 ml) were collected, and those showing high aromatic aminotransferase activity were pooled and concentrated by an Ultrafree®-15 centrifugal filter device (Millipore Corporation, Bedford, MA).

Inactivation of PheC

Construction of *phe*C*-Hg^r cassette. In order to inactivate *pheC* by gene insertion, an internal fragment of *pheC* was utilized for a strategy depending upon homologous recombination. *P. aeruginosa* is resistant to most antibiotics, so a mercury-resistance gene was used as a replacement marker since this has been successfully used to inactivate genes of the tryptophan pathway in *P. aeruginosa* (Esser *et al.*, 1990). Fig. 2-2 is the schematic representation of the construction of the vector. A 0.6-kb *Bal*I-*Stu*I fragment from *pheC* subclone pJZ1g (Zhao *et al.*, 1992) containing *pheC* lacks both 5' and 3' termini. This fragment was ligated into the *Sma*I site of pUFR004, and used to transform *E. coli* DH5α. The transformants were selected as white colonies on plates containing LB + chloramphenicol + X-gal. This pUFR004 derivative was designated as pJG-C. Strain BB1606/pDE-Hg

Fig. 2-2. Schematic representation depicting the construction of vector pJG-C_{Hg}.


pUFR004
EcoRI
SmaI
HindIII
lacZα
ori(ColE1)
Cmr
Mob(p)
pJZ1g
EcoRI
StuI
pheC
BalI
HindIII
Apr
pZ1918
PstI
HindIII
lacZ
PstI
HindIII
Apr
pDE:Hg
PstI
mer
PstI
SmaI
BalI · StuI
PstI
PstI
pJG-C
EcoRI
'pheC
HindIII
ori(ColE1)
Cmr
Mob(p)
pJG-Hg
PstI
HindIII
mer
PstI
HindIII
Apr
HindIII
HindIII
pJG-CHg
EcoRI
'pheC
HindIII
HindIII
ori(ColE1)
Cmr
Mob(p)

(Esser *et al.*, 1990) harbors a plasmid with a *mer*-operon insert. The *mer*-operon was released by *Pst*I single digestion and ligated into the *Pst*I site of pZ1918 (Schweizer, 1993), producing pJG-Hg. The *mer*-operon then was released by *Hind*III single digestion from pJG-Hg and ligated into *Hind*III site of pJG-C, producing the *phe*C*-Hg^r cassette denoted as pJG-C$_{Hg}$.

Insertional inactivation of *P. aeruginosa pheC* by pJG-C$_{Hg}$. Fig. 2-3 is the schematic representation of *pheC* disruption *in vivo*. pJG-C$_{Hg}$ was transformed into *E. coli* S17-1 (Simon *et al.*, 1983), a mobilizing donor strain, and the transformants were selected on LB agar + $HgCl_2$. *E. coli* S17-1/(pJG-C$_{Hg}$) and PAO1 were grown in LB broth to the mid-exponential phase (PAO1 at 42°C and *E. coli* S17-1/(pJG-C$_{Hg}$) at 37°C), mixed (1:1), and pelleted by centrifugation. The mating mixture was resuspended in 200 µl of LB and spread onto a nitrocellulose membrane (82.5-mm diameter, 0.45-µm pore size) positioned on a prewarmed LB agar plate. Mating mixtures were incubated for 16-20 h at 37°C, and then the cells were suspended and spread onto Pseudomonas Isolation Agar (PIA, Difco) containing mercuric chloride to select for *P. aeruginosa* Hg^r exoconjugants that were PheC$^-$.

N-terminal Amino Acid Sequencing of PhhC

Purified PhhC was denatured by sodium dodecyl sulfate (SDS) and resolved by SDS-PAGE. The protein band was

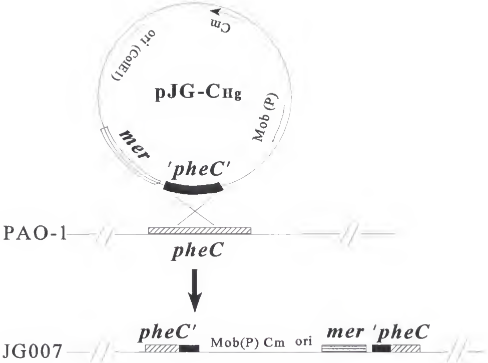


Fig. 2-3. Schematic representation of the integration of pJG-C_{Hg} into *Pseudomonas aeruginosa*.

transferred to a polyvinylidene difluoride membrane by a protein miniblotting apparatus and sequenced by use of an Applied Biosystems model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyzer by the Protein Core Facility of the Interdisciplinary Center of Biotechnology Research (ICBR) at the University of Florida.

Molecular Mass Determination

The molecular mass of the native enzyme was determined by gel filtration using an FPLC-connected Superdex®75 HR 10/30 (Pharmacia Biotech). The column was operated as previously described (under Purification) using a flow rate of 0.5 ml/min. Calibration standards were cytochrome C (M_r, 12,400), carbonic anhydrase (M_r, 29,000), bovine serum albumin (M_r, 66,000), and alcohol dehydrogenase (M_r, 150,000). V_0 was determined by blue dextran (M_r, 2,000,000). SDS-PAGE was carried out to determine the subunit molecular mass of the enzyme. Lysozyme (M_r, 14,400), soybean trypsin inhibitor (M_r, 21,500), carbonic anhydrase (M_r, 31,000), ovalbumin (M_r, 45,000), bovine serum albumin (M_r, 66,000), and phosphorylase b (M_r, 97,400) were used as molecular mass standards.

Biochemicals and Chemicals

Ampicillin, kanamycin, α-ketoglutarate, shikimate, and amino acids were purchased from Sigma Chemical Company;

mercuric chloride was from Fisher; dithiothreitol (DTT) was from Research Organics, Inc.; DEAE-cellulose was from Whatman; hydroxylapatite was from Bio-Rad. Molecular mass standards for gel filtration and for SDS-PAGE were from Sigma and Bio-Rad, respectively. Growth medium components and agar were from Difco. Chorismate, prephenate, and *L*-arogenate were made by this lab.

Results

The Presence of a Chorismate-to-phenylalanine Biosynthetic Pathway in the Periplasm

A modified magnesium shock procedure was used to separate periplasmic and cytoplasmic fractions of *P. aeruginosa* cultures grown in minimal medium. The established periplasmic enzyme PheC (Zhao *et al.*, 1993a) was used as a periplasmic protein marker and shikimate dehydrogenase was used as a cytoplasmic protein marker. It was preferable to use prephenate rather than *L*-arogenate as a substrate in the assay of PheC activity, not only because *L*-arogenate is expensive but because the assay procedure for *L*-arogenate dehydratase is tedious. However, PheC cannot be monitored with this assay in the presence of cytoplasmic P-protein because the PheA domain also utilizes prephenate. To avoid this problem, the *aroQ*$_p$-*pheA*-deficient mutant PAT1051 was used. Thus, all the prephenate dehydratase activity in PAT1051 is derived from PheC, which thus can be used as a convenient marker of a technically good separation of the periplasmic and cytoplasmic fractions. An approach is also available to accomplish the selective assay of PheC without interference from PheA in wild type PAO1. In wild type PAO1, prephenate dehydratase activity of the P-protein is completely feedback inhibited by phenylalanine, while PheC is completely insensitive. Thus, one can calculate the fraction of total activity that is contributed by each of the two enzymes by comparing activities measured in the presence and absence of *L*-phenylalanine. From Table 2-2, a calculated 91% of PheC was released into the periplasm of PAT1051, compared to 93% of PheC from PAO1. Ninety-seven percent of shikimate dehydrogenase was in the cytoplasm of PAO1, compared to 93% from PAT1051. These data affirm a technically good separation of the periplasmic and cytoplasmic fraction.

AroQ$_f$ was found in the periplasm in *P. aeruginosa* PAO1. None of the periplasmic chorismate mutase activity could be attributed to AroQ$_p$ because no PheA-domain activity was detected (Table 2-2). Hence the chorismate mutase activity in the periplasm must be exclusively AroQ$_f$. Identity of this activity as AroQ$_f$, and not AroQ$_p$ was verified by elution of a single peak of chorismate mutase in the wash fraction

Table 2-2. The presence of chorismate mutase-F and aromatic aminotransferase in the periplasmic fraction of *Pseudomonas aeruginosa*.

Fractions	Enzymes		Strains	
			PAO1	PAT1051
Periplasmic fraction	DT	Total PDT[a]	1154	574
		PheC[b]	1278	589
		PheA[c]	0	0
	AroQ$_f$		184	81
	AAT		181	244
	SDH		13	38
Cytoplasmic fraction	DT	Total PDT[a]	1373	71
		PheC[b]	50	60
		PheA[c]	1323	11
	AroQ$_p$		605	22
	AAT		862	2528
	SDH		375	478
Whole-cell extract	DT	Total PDT[a]	2706	617
		PheC[b]	1220	604
		PheA[c]	1486	13
	AroQ$_f$ + AroQ$_p$		734	88
	AAT		905	3714
	SDH		402	512

[a] Prephenate dehydratase activity assayed in the absence of *L*-phenylalanine.

[b] Prephenate dehydratase activity assayed in the presence of 1 m*M* *L*-phenylalanine.

[c] Calculated as "Total PDT" minus "PheC".

Abbreviations: DT, dehydratase; PDT, prephenate dehydratase; PheC, cyclohexadienyl dehydratase; PheA, prephenate dehydratase domain of P-protein; AroQ$_p$, chorismate mutase domain of P-protein; AroQ$_f$, monofunctional chorismate mutase; AAT, aromatic aminotransferase; SDH, shikimate dehydrogenase.

All values given are total enzyme activities (1 unit is defined as nmol of product/min).

recovered after Mono Q chromatography of the periplasmic fraction. This is the established elution behavior of AroQ_f. About 20% of total aromatic aminotransferase activity was found in the periplasm, and it was surprising to find by use of Mono Q chromatography that the aromatic aminotransferase activity was distributed into three well-separated peaks.

Partial Purification of AroQ_f and an Aromatic Aminotransferase from *Pseudomonas aeruginosa* Periplasm

AroQ_f was partially purified from the periplasmic fraction of PAO1. The purification steps are summarized in Table 2-3. After the last purification step, one distinctive band with a molecular mass of around 22,000 was revealed by SDS-PAGE after staining with Coomassie blue (Fig. 2-4). The native molecular mass estimated by gel filtration was also around 22,000, thus suggesting a native monomer structure.

Aromatic aminotransferase from the periplasmic fraction of PAO1 revealed three separated peaks after Mono Q chromatography during a preliminary analytical study. For purification on a larger scale, hydroxylapatite chromatography was used as the first step. Two separated aromatic aminotransferase peaks were eluted in the gradient (Fig. 2-5), denoted AAT-1 and AAT-2, respectively. These were pooled separately and loaded onto a Mono Q column. AAT-1 was recovered as a single peak in the gradient (Fig. 2-5). AAT-2

Table 2-3. Purification of the monofunctional chorismate mutase (AroQ$_f$) from *Pseudomonas aeruginosa*.

Purification step	Total protein (mg)	Specific activity[a]	Purification (fold)	Yield (%)
Shock fluid	240	17.7	1	100
Hydroxylapatite	39.9	39.2	2.2	36.8
Mono Q (pH 7.0)	10.5	53.1	3	13.1
Mono Q (pH 8.4)	1.7	116.5	6.6	4.7
Superdex® 75	0.44	487.5	27.5	0.4

[a] Specific activity expressed as nmol min^{-1} mg^{-1}.

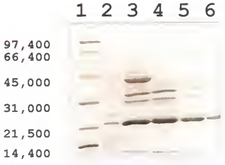
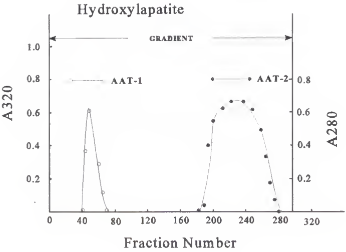
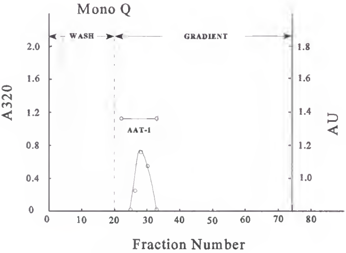
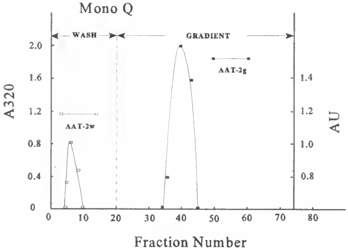


Fig. 2-4. SDS-PAGE of AroQ$_f$ from *Pseudomonas aeruginosa*. The protein samples were separated in a 12% gel and stained with Coomassie blue. Lane 1: molecular weight standards; Lane 2: the periplasmic fraction; Lane 3: the fraction collected after hydroxylapatite chromatography; Lane 4: the fraction collected after Mono Q (pH 7.0) chromatography; Lane 5: the fraction collected after the second Mono Q (pH 8.4) chromatography; Lane 6: the fraction collected after Superdex 75 chromatography.

Fig. 2-5. The periplasmic aromatic aminotransferase elution profiles on i) hydroxylapatite chromatography (upper panel), AAT-1 eluted at about 0.10 *M* phosphate concentration, AAT-2 eluted at about 0.35 *M* phosphate concentration; ii) Mono Q chromatography (middle panel) for AAT-1, eluted at about 0.2 *M* KCl; iii) Mono Q chromatography (lower panel) for AAT-2g, eluted at about 0.4 *M* KCl. Dotted lines represent the protein eluate profile. A320: absorbance at 320 nm; A280: absorbance at 280 nm; AU: absorbance unit determined by FPLC system at 280 nm.


Hydroxylapatite
GRADIENT
AAT-1
AAT-2
A320
A280
1.0
0.8
0.6
0.4
0.2
0
40
80
120
160
200
240
280
320
Fraction Number


Mono Q
WASH
GRADIENT
AAT-1
A320
AU
2.0
1.6
1.2
0.8
0.4
0
1.8
1.6
1.4
1.2
1.0
10
20
30
40
50
60
70
80
Fraction Number


Mono Q
WASH
GRADIENT
AAT-2w
AAT-2g
A320
AU
2.0
1.6
1.2
0.8
0.4
0
1.4
1.2
1.0
0.8
10
20
30
40
50
60
70
80
Fraction Number

split into AAT-2w in the wash eluate and AAT-2g in the gradient eluate (Fig. 2-5). At this point, the protein amounts of AAT-1 and AAT-2w were so low that no distinctive bands were visualized on SDS-PAGE, though their native molecular mass could be determined from Superdex 75 chromatography as 42 kd for both AAT-1 and AAT-2w. Only AAT-2g was recovered successfully following purification (Table 2-4). It exhibited a subunit molecular mass of 34,000 as estimated by SDS-PAGE staining with Coomassie blue (Fig. 2-6). Since the native molecular mass estimated by gel filtration was around 64,000, AAT-2g must be a homodimer.

Complementation of *E. coli* Aminotransferase Deficiencies by *phhC*

It has been reported that *P. aeruginosa* possesses five aromatic aminotransferases (Whitaker *et al.*, 1982). *phhC,* encoding an aminotransferase located within a three-component gene cluster (Zhao *et al.*, 1994), was sequenced and is a homolog of *E. coli* TyrB and AspC, aminotransferases that can function with aromatic substrates. PhhC was characterized because of the particular possibility it might be a periplasmic aromatic aminotransferase, and if not, because of the general possibility that insight would be gained about the individual roles of the multiple aromatic aminotransferases. Plasmid pJS3A, a pUC18 derivative possessing *phhB* and *phhC,*

Table 2-4. Purification of the aromatic aminotransferase (AAT-2g) from *Pseudomonas aeruginosa*.

Purification step	Total protein (mg)	Specific activity[a]	Purification (fold)	Yield (%)
Shock fluid	240	67.1	1	100
Hydroxylapatite[b]	16	235.5	3.5	23.4
Mono Q (pH 7.0)[c]	0.88	1568.7	23.3	8.6[d]
Superdex® 75	0.47	4440.5	66.2	1

[a] Specific activity given as nmol min^{-1} mg^{-1}.
[b] Peak AAT-2 (see Fig. 2-5).
[c] Peak AAT-2g (see Fig. 2-5).
[d] Note that the yield is underestimated because previous fractionation steps included two additional aminotransferases.

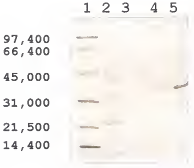


Fig. 2-6. SDS-PAGE of periplasmic aromatic aminotransferase (AAT-2g) from *Pseudomonas aeruginosa*. The protein samples were separated in a 12% gel and stained with Coomassie blue. Lane 1: molecular weight standards; Lane 2: the periplasmic fraction; Lane 3: the fraction collected after hydroxylapatite chromatography; Lane 4: the fraction collected after Mono Q chromatography; Lane 5: the fraction collected after Superdex 75 chromatography.

was transformed into DL39 (LeMaster and Richards, 1988), an *E. coli* strain which is an *aspC tyrB ilvE* aminotransferase triple mutant, requiring aspartate, tyrosine, phenylalanine, leucine and isoleucine for growth. Transformant colonies were removed from LB + ampicillin plates, suspended in minimal medium, and then spread on minimal plates. Sterile discs containing different combinations of amino acids were placed on the plates. After incubation, growth occurred around the discs containing branched-chain amino acids (Leu + Ile), while there was no growth when branched-chain amino acids were absent (Table 2-5). This observation, as anticipated, shows that PhhC, *in vivo*, can replace aspartate aminotransferase (AspC) and aromatic aminotransferase (TyrB), but not branched-chain aminotransferase (IlvE).

Crude extract prepared from DL39/pJS3A possessed very high aromatic aminotransferase activity, while crude extract made from DL39/pJS3B lacked detectable activity. This suggested that there was either no promoter present in pJS3A since the first gene, *phhA* of the three-component cluster was truncated, or if an internal promoter exists for *phhC*, it could not be recognized in *E. coli*.

Purification and Characterization of PhhC in *E. coli*

The *P. aeruginosa* aromatic aminotransferase was purified from *E. coli* DL39/pJS3A by use of three chromatography steps

Table 2-5. Ability of *phhC* to complement deficiencies of *aspC, tyrB* or *ilvE* in *E. coli* DL39 containing pJS3A.

TEST DISC	SUPPLEMENTATION			GROWTH
	TYR + PHE	ASP	ILE + LEU	
A	+	+	+	+
B	−	+	+	+
C	+	−	+	+
D	+	+	−	−

The medium was minimal + ampicillin, see Materials and Methods.

(Table 2-6). The cloned PhhC showed a heterogeneous elution profile from a hydroxylapatite column (Fig. 2-7A), yielding a leading peak in the wash fraction and a trailing peak in the gradient fraction. Protein from either peak, when reloaded onto the same hydroxylapatite column and eluted under the same conditions, eluted with the same bi-peaked profile (Fig. 2-7B,7C). All three of the column profiles shown in Fig. 2-7 were similar. The leading peak was broad, consistent with a new equilibrium of charge forms occurring during chromatography. PhhC collected from either of the two peak positions showed no difference in molecular weight as estimated by gel filtration. Each had identical N-terminal amino acid sequences (SHFAKVARVP). These corresponded exactly with the N-terminal amino acid sequences published by Zhao *et al.* (1994), except that the starting methionine had been processed. The PhhC collected from the wash fraction had fewer contaminating proteins than that from gradient fraction (Fig. 2-8), and this fraction was used for the final step on Superdex 75 gel filtration, a single protein band was observed after SDS-PAGE (Fig. 2-8). The apparent molecular weight of this band was estimated to be 43,000 which was in good agreement with that deduced from the DNA sequence, and confirmed by mass spectrometry (43,276) of pure protein. The molecular mass of the native enzyme was 52,000 Da, on the criterion of gel filtration on Superdex 75.

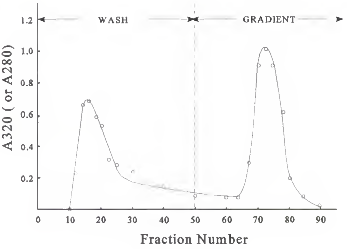
Table 2-6. Purification of the cloned *P. aeruginosa* aromatic aminotransferase (PhhC) from *E. coli* DL39/pJS3A.

Purification Step	Total protein (mg)	Specific activity[a]	Purification (factor)	Yield (%)
Crude extract	1184	1.7	1	100
DEAE-cellulose	320	3.5	2	55.6
Hydroxylapatite				
(Gradient)	40	11.9	7	23.6
(Wash)	5	24.5	14.4	6.1
Superdex® 75[b]	1	32.1	18.9	1.6

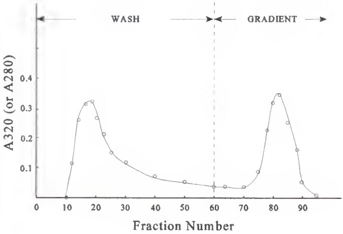
[a] Specific activity given as nmol min^{-1} mg^{-1}.
[b] The protein recovered from the wash fraction of hydroxylapatite (see fig. 2-7) was applied.

Fig. 2-7. PhhC hydroxylapatite elution profile. A) The protein applied was from the DEAE-cellulose step of purification (Table 2-6); B) The loaded protein was from the wash fraction of A; C) The loaded protein was from the gradient fraction of A. All the gradient peaks eluted at about 0.1 *M* phosphate concentration. Dotted lines represent protein profile. A320 or A280: absorbance at 320 nm or 280 nm.

A


WASH
GRADIENT
A320 (or A280)
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
90
Fraction Number

B


WASH
GRADIENT
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0.3
0.2
0.1
0
10
20
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40
50
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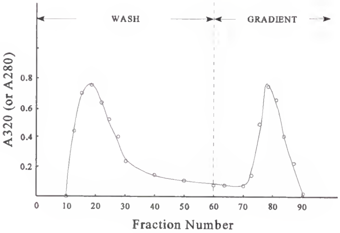
70
80
90
Fraction Number

C


WASH
GRADIENT
A320 (or A280)
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0.6
0.4
0.2
0
10
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60
70
80
90
Fraction Number

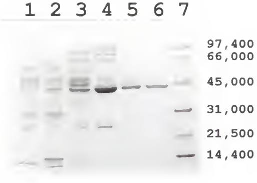


Fig. 2-8. SDS-PAGE of aromatic aminotransferase (PhhC) from *E. coli* DL39/pJS3A. The protein samples were separaed in a 12% gel and stained with Coomassie blue. Lane 1: the crude extract of DL39/pUC18; Lane 2: the crude extract of DL39/pJS3A; Lane 3: the fraction collected after DE-52 chromatography; Lane 4: the gradient fraction collected after hydroxylapatite chromatography; Lane 5: the wash fraction collected after hydroxylapatite chromatography; Lane 6: the fraction collected after Superdex 75 chromatography; Lane 7: molecular weight standards.

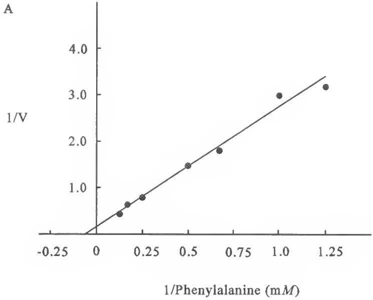
The K_m and V_{max} values (Table 2-7) for *L*-phenylalanine and *L*-tyrosine were calculated from the double-reciprocal plots (Fig. 2-9) of initial velocity as a function of the varied concentration of *L*-phenylalanine or *L*-tyrosine when α-ketoglutarate was maintained at saturating concentration. From the V_{max}/K_m ratio, it seems that PhhC favors tyrosine.

Inactivation of PheC

In order to evaluate the functional role of the periplasmic pathway of phenylalanine biosynthesis, *pheC* was interrupted by *in vivo* gene insertion. A ColE1-based suicide vector pUFR004 (Defeyter *et al.*, 1990) can propagate in *E. coli*, but not in *P. aeruginosa*. When such a vector is introduced into *P. aeruginosa*, no phenotype dictated by the marker gene on the vector will be conferred unless the vector is incorporated into the host chromosome. *pheC*, truncated at both N- and C-termini, was ligated into the pUFR004, as was the mercury-resistance gene, giving the plasmid pJG-C_{Hg}. This plasmid was transferred into PAO1 by conjugation with the help of a mobilizing donor strain *E. coli* S17-1 (Simon *et al.*, 1983), as described in Materials and Methods. *P. aeruginosa* PheC$^-$ Hg^r exconjugants were selected on the Pseudomonas Isolation Agar containing mercuric chloride. Clone JG007 was used as a source of culture forextract preparation in order to

Table 2-7. Kinetic parameters of PhhC for aromatic amino acid substrates.

Varied substrate	K_m (mM)	V_{max}	V_{max}/K_m
Phenylalanine	14.3	5.7	0.4
Tyrosine	1.18	1	0.8



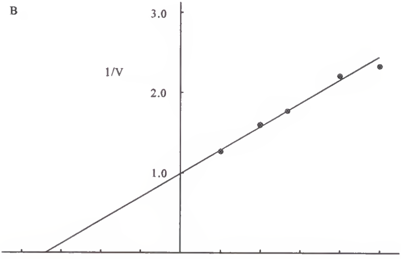


Fig. 2-9. Double reciprocal plots of initial velocity as a function of the substrate phenylalanine (A) or tyrosine (B). The fixed substrate was 10 m*M* α-ketoglutarate.

assay PheC activity. It is clearly shown by the data in Table 2-8 that the cyclohexadienyl dehydratase in JG007 has been inactivated. Thus, when PheC was assayed with use of *L*-arogenate as substrate, no activity was found in the periplasmic fraction of JG007, in contrast to PAO1. Alternatively, when PheC was assayed with prephenate as substrate, no phenylalanine-resistant activity was found in the periplasm of JG007, in contrast to results obtained with PAO1.

Phenotype of JG007

There is no obvious growth-rate difference between wild type PAO1 and mutant JG007 in glucose minimal medium. This is not surprising, since one does not expect the periplasmic pathway to provide phenylalanine for cell growth under normal conditions.

P. aeruginosa is sensitive to the *L*-tyrosine analogue *m*-fluoro-tyrosine (MFT) and to the *L*-phenylalanine analogue *p*-fluoro-phenylalanine (PFP) on fructose-based minimal medium, but not on glucose-based minimal medium. The explanation for this phenomenon is that glucose supports an elevated endogenous *L*-phenylalanine pool within the cells. Is the unregulated periplasmic pathway responsible for the elevated endogenous *L*-phenylalanine pool during growth on glucose? When PAO1 (wildtype control) and JG007 were spread on the

Table 2-8. Lack of PhhC activity in JG007 (total units, 1 unit is defined as nmol/min).

Strain	Fraction	PDT[a]		ADT[a]
		-PHE	+PHE	
PAO1	periplasmic[b]	929	879	190
JG007	periplasmic	0	0	0
PAO1	cytoplasmic[b]	426	0	0
JG007	cytoplasmic	400	0	0

[a] PheC was assayed with its alternative substrates, prephenate or *L*-arogenate. Prephenate dehydratase (PDT) was assayed by measuring the absorption of phenylpyruvate at 320 nm, and arogenate dehydratase (ADT) was assayed by measuring the formation of phenylalanine from *L*-arogenate using HPLC.

[b] As a cytoplasmic marker control, shikimate dehydrogenase was found in the cytoplasmic fraction but not in the periplasmic fraction (data not shown).

glucose-based minimal plate on which a sterile disc containing MFT and PFP had been placed, no inhibition zone was found around these discs in either strain. Thus, JG007 is not hypersensitive to *L*-phenylalanine analogue. Blocking of the periplasmic phenylalanine pathway by inactivation of *pheC* did not decrease the level of the endogeneous *L*-phenylalanine pool, thereby triggering analogue sensitivity.

PheC has been reported to be a homologue of the LAO binding protein from *E. coli* (Tam and Saier, 1993). When PAO1 and JG007 were inoculated in minimal medium with *L*-lysine or *L*-arginine as the sole carbon source, both strains grew well in either medium. This indicated that PheC is not the functional equivalent of the LAO binding protein in *P. aeruginosa*.

Attempt to Clone *aroQ*$_f$ from *P. aeruginosa*

An effort has been made to clone *P. aeruginosa aroQ*$_f$ in *E. coli* by functional complementation. *E. coli* double mutant JP2255 which is deficient in *aroQ*$_p$*-pheA* and *aroQ*$_t$*-tyrA* is required to give a genetic background lacking chorismate mutase activity. However, an *aroQ*$_f$ clone will not give a selectable phenotype (i.e., growth on minimal medium in the presence of *L*-phenylalanine alone or *L*-tyrosine alone), because the double mutant lacks the dehydratase and dehydrogenase activities which are necessary to complete the overall conversion of chorismate to phenylalanine or tyrosine, respectively. To solve this problem and also to make the best use of our existing *P. aeruginosa* PAO1 genomic library constructed in pUC18, a plasmid derivative of pACYC177 has been constructed. pACYC177 is a multicopy plasmid containing the replication system of miniplasmid P15A (Chang and Cohen, 1978). This plasmid is compatible with ColE1-derived replicons, such as pUC18, and can be propagated concurrently with such replicons in the same bacterial cell. A DNA fragment containing cyclohexadienyl dehydrogenase gene, *tyrC* from *Zymomonas mobilis* (Zhao *et al.*, 1993b) was inserted into *Hinc*II site of pACYC177, disrupting the gene encoding ampicillin-resistance. This pACYC177 derivative, denoted pJG-T, was then transformed into JP2255. This strain, denoted JP2255-T, should be suitable as a host to screen a PAO1 genomic library for *aroQ*$_f$ clones on minimal medium containing ampicillin, kanamycin, thiamine, phenylalanine, and shikimate. Although many attempts have been made, I failed to get an *aroQ*$_f$ clone.

Discussion

Functional Role of the Periplasmic Pathway

P. aeruginosa PheC has been inactivated *in vivo*. The mutant JG007 does not differ from wild type in terms of the endogeneous phenylalanine pool maintained. It cannot be the *P. aeruginosa* equivalent of the *E. coli* LAO binding protein since it utilizes *L*-arginine or *L*-lysine normally as carbon sources. PheC does seem to be involved in *L*-arogenate utilization, because JG007 grows more slowly than wildtype in minimal medium with *L*-arogenate as the sole source of nitrogen (Fischer *et al.*, 1997).

The localization of this pathway in the periplasmic space makes one wonder if the genes involved are regulated under environmental stress. Dr. Deretic of University of Michigan has been working on *P. aeruginosa* mucoidy in cystic fibrosis extensively (Deretic *et al.*, 1994). His group identified a cluster of three tightly linked genes which controlled development of mucoid phenotype (Martin *et al.*, 1993). One of the genes, *algU*, was a homologue of the alternative sigma factor (σ^H in *Bacillus* and σ^E in *E. coli*) (Martin *et al.*, 1994), and was required for the transcriptional activation of *algD*, a critical event in the establishment of mucoidy. Two other genes (*mucA-mucB*) in the cluster counteracted the activity of *algU*, so when these genes were present, strain (PAO381) remained non-mucoid. When one of them (*mucB*) was insertionally inactivated, strain (PAO671) resulted in mucoid phenotype and a strong transcriptional activation of *algD*. PAO381 and PAO671, supplied by Dr. Deretic, were assayed to determine the levels of the three periplasmic enzymes. No significant difference was found between the two strains, with respect to the levels of PAO1. This ruled out that the periplasmic pathway to *L*-phenylalanine is regulated by σ^E. If that were the case, one would expect the enzyme levels in PAO671 would be elevated. We recently receieved another pair of *P. aeruginosa* strains, *rpoN*$^-$ and its parent strain. *rpoN* encodes for a σ^{54} type of transcriptional initiator. It will be worthwhile to check the periplasmic enzyme activity levels in these two strains to see if the expression of the pathway is regulated by σ^{54}.

The longtime laboratory K-12 strain of *E. coli* we used lacks both periplasmic enzymes. However, might other *E. coli* strains in nature possess the periplasmic enzymes? This seemed possible since closely related enteric bacteria possess active periplasmic enzyme activities. We explored the possible existence in recently isolated enteropathogenic *E. coli* (EPEC)(Girón *et al.*, 1993), of the periplasmic enzymes. However, EPEC strains B171, B171-8, and O127:H6 and BFP negative EPEC strain B171-5, kindly provided by Dr. Schoolnik of Stanford, showed no detectable activity of $AroQ_f$ or PheC.

Genes upstream of *pheC* proved to belong to the *rhlABRI* gene cluster involved in rhamnolipid biosynthesis and its regulation (Ochsner and Reiser, 1995; and Brint and Ohman, 1995). Rhamnolipid is believed to be a virulence factor excreted by *P. aeruginosa*, and the regulation of its synthesis employs cell-density dependent control, as exemplified by the LuxR-LuxI system in *Vibrio fischeri* (Fuqua *et al.*, 1994) and LasR-LasI system in *P. aeruginosa* (Gambello and Iglewski, 1991; and Passador *et al.*, 1993). It seemed possible that *pheC* might also be regulated by RhlR-RhlI since *pheC* is immediately downstream of *rhlR-rhlI*. Rhamnolipid-deficient *rhlR* and *rhlI* mutants, kindly provided by Dr. Ochsner, were analyzed for possible effect upon PheC, but no effect was observed. It therefore seems that *pheC* is not a member of this quorum-sensing regulatory system.

L-Phenylalanine is a precursor of *Pseudomonas* fluorescent pigment pyoverdin, which is a siderophore involved in iron uptake (Maksimova *et al.*, 1992). Could it be that the periplasmic pathway provides precursor phenylalanine residue for the chromophore portion of pyoverdin? Since *P. aeruginosa* produces siderophores other than pyoverdin for iron uptake (i.e., pyochelin, salicylate) (Serino *et al.*, 1995), a periplasmic-pathway linkage to pyoverdin formation might not be easily shown. A slower growth rate of JG007 on iron-limiting medium might be observed with (i) introduction of mutations blocked in pyochelin and salicylate production or (ii) use of a stressed growth condition which limits the pool of chorismate (i.e., fructose as carbon source, use of glyphosate).

Roles of the Multiple Aromatic Aminotransferases in *P. aeruginosa*

Five separable species of aromatic aminotransferase have been reported in *P. aeruginosa* (Whitaker *et al.*, 1982). Relatively small differences in substrate specificity were noted. In spite of this, evidence exists to suggest non-overlapping functional roles. Thus, mutant NP-72 lacks one of the aminotransferases and is a phenylalanine bradytroph (Whitaker *et al.*, 1982). RW8-27 is a double mutant which carries a second aminotransferase deficiency in the NP-72 genetic background; it is a tight phenylalanine auxotroph (Whitaker *et al.*, 1982). Thus, the remaining aminotransferases are unable to perform phenylalanine biosynthesis *in vivo*. It is possible that the double mutant is also unable to synthesize tyrosine, but that the deficiency is masked in the presence of exogenous phenylalanine by the activity of phenylalanine hydroxylase (Zhao *et al.*, 1994). On the other hand, a PhhC-deficient mutant cannot use phenylalanine or tyrosine as a carbon source, and therefore PhhC is dedicated to catabolism. Preliminary data indicate that PhhA and PhhB form a complex (Song, unpublished data). Since PhhB and PhhC are translationally coupled, a tripartite complex may exist in vivo, effectively specializing PhhC for catabolic function.

Surprisingly, three species of aromatic aminotransferase were separated from the periplasmic fraction of *P. aeruginosa*. In the assay condition we used, any periplasmic or membrane-bound oxidase might oxidize *L*-phenylalaine in the reaction mixture to phenylpyruvate and give a spectrometer reading. This interference was ruled out by the control reaction containing no α-ketoglutarate. Thus, all the activities we found were from aromatic aminotransferases. It is not known whether any of these correspond to PhhC or to either of the two aminotransferases that were eliminated in strain RW8-27. An analysis of periplasmic and cytoplasmic fractions from mutants NP-72, RW8-27, and JS104 with the methodology established in this thesis would answer this question.

One possibility under consideration is that only one truly periplasmic aminotransferase exists. The remaining two species are charge isoforms of PhhC (see Discussion below). Since phenylalanine hydroxylase uses molecular oxygen, the phenylalanine hydroxylase complex might be loosely associated with the inner membrane in order to have access to the more oxygenating environment of the periplasm. Membrane-associated proteins that are not located within the periplasm are proven to be released into periplasmic fraction following techniques such as osmotic shock. If correct, then two aminotransferases (defined by strain RW8-27) might be associated with phenylalanine and tyrosine biosynthesis in the cytoplasm, PhhC (represented by two charge isomers) might be associated with a membrane-linked catabolic system, and a single aromatic aminotransferase may function with the periplasmic pathway of phenylalanine biosynthesis. The native molecular weights estimated for species AAT-1 and AAT-2w (42 kDa), as resolved in Fig. 2-5, would fit expectations for the charge isomers of PhhC (see below). The third periplasmic aminotransferase species (AAT-2g) has a molecular mass of 34 kDa. As such, it must not be a member of the Family-I aminotransferases (Jensen and Gu, 1996).

Apparent Charge Isoforms of PhhC

Although PhhC may prove to be recoverable in periplasmic fraction as discussed above, it does not have an obvious cleavable signal peptide on the criterion of computer analysis (e.g., P-sort). More directly, the N-terminal amino acid sequence of purified protein corresponded exactly with the N-terminus deduced from the DNA sequence. The two separable species of PhhC obtained had to originate from a single gene since it was purified from the cloned gene in *E. coli*. The possibility that interconvertible molecular-weight forms of the enzyme might be in equilibrium was considered. However, each separated form eluted at identical molecular-weight position following gel filtration. Since the gene encoding PhhB (carbinolamine dehydratase) coexists with PhhC in the clone used for expression, a strong possibility seemed to be that one peak of activity might represent a PhhB/PhhC complex, the other a dissociated PhhC, [the low molecular weight of PhhB (13 kDa) might account for ambiguity in discriminating molecular-weight differences]. However, this would predict that enzyme in the latter peak would rechromatograph in the same position instead of the bi-peaked elution profile obtained. Furthermore, no PhhB was detected under either peak when antibody to PhhB was used in Western analysis (data not shown). Another possible explanation was some form of covalent modification, e.g., phosphorylation. However, it seems unlikely that each form of the enzyme would yield a new equilibrium mixture when rechromatographed. Thus, I conclude that an equilibrium of PhhC charge isoforms exists which are readily discriminated by the hydroxylapatite matrix.

PhhC belongs to the Family-I aminotransferase homology group (Jensen and Gu, 1996). This family shares a mechanism of catalysis which requires homodimer formation. Following gel filtration, a native molecular weight (of both isoforms) was obtained which was much closer to a monomeric species than to the expected dimeric species. The possibility that substrates were required to stabilize the dimeric state was considered. However, exactly the same gel-filtration results were obtained when a column was run in the presence of α-ketoglutarate and *L*-phenylalanine. Therefore, it was concluded that some property of PhhC interferes with an accurate molecular-weight determination.

PhhC appears to be an essential enzyme for *L*-tyrosine catabolism (Song, unpublished data), the results, showing a preference of PhhC for *L*-tyrosine over *L*-phenylalanine, is consistent with its probable role *in vivo* for transamination of *L*-tyrosine to 4-hydroxyphenylpyruvate. Among homologs of PhhC, AspC and TyrB from *E. coli* have been studied to determine the variation of catalytic residues which influence whether L-aspartate or aromatic amino acids are favored as substrates (Onuffer and Kirsch, 1995). Although PhhC is clearly dedicated to function with aromatic substrates, the pattern of critical catalytic residues resembles AspC more than TyrB. It has been found that *phhC* can complement *E. coli* deficiencies in both *aspC* and *tyrB*. A fuller characterization of the substrate profile of PhhC is underway.

Perspective of Future Study

An alternative route of phenylalanine biosynthesis was shown to be present in the periplasmic space of *P. aeruginosa*.

This route comprises monofunctional chorismate mutase (AroQ$_f$), cyclohexadienyl dehydratase (PheC), and aromatic aminotransferase. AroQ$_f$ and one species of periplasmic aromatic aminotransferase were purified extensively. The low yields obtained would be insufficient for preparation of polyclonal antibody. However, they should be sufficient for preparation of monoclonal antibody, which can be utilized for various Western blot analyses, for the immunogold electron microscopic evaluation of spatial compartmentation, and for the cloning of the *P. aeruginosa* genes encoding *AroQ$_f$* and periplasmic aminotransferase.

CHAPTER 3
THE AROQ AND PHEA DOMAINS OF THE BIFUNCTIONAL P-PROTEIN FROM *XANTHOMONAS CAMPESTRIS*

Introduction

The chorismate mutase reaction is a universal step of carbon-flow commitment to biosynthesis of *L*-phenylalanine and *L*-tyrosine. Subsequent enzymatic steps vary in nature (Jensen, 1992). For the *L*-phenylalanine pathway, prephenate can be transaminated to *L*-arogenate via prephenate aminotransferase and then converted to *L*-phenylalanine via arogenate dehydratase. Alternatively, prephenate can be converted to phenylpyruvate via prephenate dehydratase and then transaminated to *L*-phenylalanine. Some organisms possess a broad-specificity enzyme, cyclohexadienyl dehydratase, which is competent for catalysis of both the prephenate dehydratase and arogenate dehydratase reactions.

Two of the three major divisions of gram-negative bacteria possess a bifunctional 'P-protein' that carries two catalytic domains, one specifying chorismate mutase ($AroQ_p$) and another specifying prephenate dehydratase ('PheA). This presumably arose long ago by gene fusion prior to the divergence of the two gram-negative divisions (Ahmad and Jensen, 1986). Much more recently, a bifunctional 'T-protein' emerged in enteric bacteria. This carries two catalytic domains, one specifying chorismate mutase ($AroQ_t$) and the other prephenate dehydrogenase (TyrA). Chorismate mutase may also exist as a monofunctional protein ($AroQ_f$). In at least some gram-negative bacteria, $AroQ_f$ is processed for translocation to the periplasm (Xia *et al.*, 1993b). Many enteric bacteria possess $AroQ_p$, $AroQ_t$ and $AroQ_f$ as coexisting isoenzyme species (Xia and Jensen, 1992).

In this context the P-protein encoded by *aroQ*$_p$-*pheA* will yield interesting comparative information and an ever-more informative evolutionary perspective as representative genes are cloned in an appropriately spaced phylogenetic progression. To date *aroQ*$_p$-*PheA* has been cloned from enteric bacteria [*Escherichia coli* (Hudson and Davidson, 1984), and *Erwinia herbicola* (Xia *et al.*, 1993a)], sequenced from the closely related *Haemophilus influenzae* (Fleischmann *et al.*, 1995), and cloned from the more distant *Pseudomonas stutzeri* (Fischer *et al.*, 1991).

In this paper *aroQ*$_p$-*pheA* from *Xanthomonas campestris* was chosen for cloning because of its phylogenetic distance from other organisms from which *aroQ*$_p$-*pheA* has been cloned. In addition *X. campestris* represents an enzymological-patterning group different than any of the others (Whitaker *et al.*, 1985). It lacks *aroQ*$_t$-*tyrA* (unlike enteric bacteria and *H. influenzae*) but possesses *pheC* (encodes cyclohexadienyl dehydratase) and *aroQ*$_f$ (unlike *P. stutzeri*).

Materials and Methods

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this study are listed in Table 3-1, and Table 2-1. Media and supplements refer to Materials and Methods in Chapter 2, with gentamicin at 3 µg/ml, and X-gal at 40 ng/ml.

DNA Manipulation

Standard molecular biology procedures were performed as described by Maniatis *et al.* (1989) unless otherwise indicated. A genomic library of *X. campestris* was constructed by partially digesting genomic DNA with *Sau*3A and cloning into the *Bam*HI site of cosmid pUFR043. Restriction enzymes, ligase, and calf intestine alkaline phosphatase were purchased from New England Biolabs or Promega, and were used according to Manufacturers' instructions.

DNA Sequencing and Data Analysis

Plasmid pJG-XP2a was purified by the method recommended in User Bulletin 18 offered by Applied Biosystems, Inc.

Table 3-1. Bacterial strains and plasmids.

Strain/plasmid	Relevant characteristics	Source and reference
E.coli		
JM83	F$^-$, *ara*Δ*(proAB-lac)*, *rpsL*, *thi-1*, ϕ80d*lacZ*Δ*M15*	Bethesda Research Laboratories
KA197	*thi-1*, *pheA97*, *relA1*, *spoT1*	CGSC 5243
BL21(DE3)	F$^-$, *ompT*, r$_B^-$, m$_B^-$	Novagen
Plasmid		
pUC19	Ap^r, *lacI'POZ'*	Yanisch-Perron *et al.*, 1985
pUFR043	cos, Nm^r, Gm^r	Dean W. Gabriel
pET-11a	Ap^r, T$_7$ *lac*	Novagen
pka06	original clone of *pheA* from *X. campestris* in pUFR043	Dean W. Gabriel
pJG-XP1	2.7-kb *Eco*RI-*Eco*RI fragment from pka06 subcloned in pUC18	This study
pJG-XP2a	1.7-kb *Pst*I-*Eco*RI fragment from pJG-XP1 subcloned in pUC18	This study
pJG-XP2b	1.7-kb *Pst*I-*Eco*RI fragment from pJG-XP1 subcloned in pUC19	This study
pJG-XP3	1.0-kb *Pst*I-*Pst*I fragment from pJG-XP1 subcloned in pUC18	This study
pJG-XP4	1.5-kb *Pst*I-*Sac*II fragment from pJG-XP2a subcloned in pGEM-5Zf(+)	This study
pJG-XP5	1.2-kb PCR generated *pheA* subcloned in *Hinc*II site of pUC18	This study
pJG-XP6	1.2-kb *Nde*I-*BamH*I fragment from pJG-XP5 subcloned in pET-11a	This study

(Foster City, CA). Double-stranded plasmid DNA was sequenced in both directions by the DNA Sequencing Core Facility of the Interdisciplinary Center of Biotechnology Research (ICBR) at the University of Florida. In order to sequence DNA beyond the range of the T7 and M13 primers which flank the multiple cloning site of the plasmid vector pUC18, 18-mer oligonucleotides were synthesized to regions upstream of the unsequenced regions by the DNA Synthesis Core Laboratory of ICBR, UF. The nucleotide sequence and the deduced amino acid sequence were analyzed by using the updated version (Version 8.0, 1994) of the sequence analysis software package offered by Genetics Computer Group, Inc. (Devereux *et al.*, 1984).

Crude Extract Preparation and Enzyme Assays

Crude extract was prepared as described previously (Gu *et al.*, 1995). Chorismate mutase and prephenate dehydratase activities of the cloned *pheA* gene product were assayed by a modification (Ahmad and Jensen, 1988) of the method of Cotton and Gibson (1965). Protein concentration was determined by the method of Bradford (1976).

DEAE-cellulose Chromatography

Proteins in the crude extract (1.76 g), made in a standard buffer which contained 50 m*M* potassium phosphate buffer (pH 7.0), 1 m*M* *L*-tyrosine, 1 m*M* ß-mercaptoethanol, 0.1

mM PMSF (phenylmethylsulfonyl fluoride), and 20% (v/v) glycerol, was precipitated with $(NH_4)_2SO_4$ at 50% of saturation. The resuspended protein was dialyzed against 2000 ml of buffer overnight with one change of the same buffer. A 640-mg amount of protein was applied to a 2.5 x 50 cm DE-52 column equilibrated in buffer. The column was washed with 280 ml of buffer, and bound protein was then eluted with a 1000-ml linear salt gradient (0-0.3 *M* KCl) prepared in the same buffer. Fractions (5 ml) were collected and assayed for both chorismate mutase and prephenate dehydratase activities.

Hydroxylapatite Chromatography

A 30-mg amount of protein partially purified from DE-52 was loaded onto the bed of a 1.5 x 20 cm column of Bio-Gel HTP previously equilibrated with buffer. The column was washed with 60 ml of starting buffer and bound proteins eluted by application of a gradient of between 200 m*M* to 600 m*M* of potassium phosphate containing the other supplements of standard buffer except PMSF. Fractions (2 ml) were collected, and both activities were assayed.

Overexpression of *aroQ*$_p$*-pheA*

Two specially designed 29-mer oligonucleotides synthesized by the DNA Synthesis Core Laboratory of ICBR (5'-ATGCGCTGCTCAGGGAATGGCGACCGGAT-3' and 5'-

CC**CATATG**GCTCCCAAGCCCAAGAACACC-3'), provided regions spanning the entire *aroQ*$_p$*-pheA* open reading frame. The latter contains a *Nde*I site (indicated above with bold letters) overlapping the start codon of *aroQ*$_p$*-pheA*. The 1,200 bp *aroQ*$_p$*-pheA* fragment was amplified by PCR (Perkin Elmer Cetus, Norwalk, CT), and ligated into the *Hinc*II site of pUC18(pJG-XP5). The *aroQ*$_p$*-pheA* fragment was then released by double digestion with *Nde*I and *Bam*HI and ligated into pET-11a(pJG-XP6), a translation "overexpression" vector (Novagen, Inc.). Thus, *aroQ*$_p$*-pheA* was placed under control of a T7 promoter and a Shine-Delgarno sequence from the vector.

Plasmid pJG-XP6 was transformed into *E. coli* BL21(DE3). A loopful of culture from the transformed plate was inoculated into 10 ml of LB medium containing ampicillin and shaken at 37°C until the OD_{600nm} reached 0.6-1.0. IPTG was added to a final concentration of 1 m*M*, and the incubation was continued for 3 hours. The resulting culture was checked for the level of *aroQ*$_p$*-pheA* expression by SDS-PAGE.

Electron Microscopy of E. coli BL21(DE3)/pJG-XP6

Intact cells of *E. coli* BL21(DE3)/pJG-XP6 and pellets resulting from these cell lysis by sonication were observed under a Zeiss EM-10 CA transmission electron microscope by the EM lab in the Department of Microbiology and Cell Science at the University of Florida. The antibody used for immunogold labeling was the one raised against the P-protein from *Acinetobacter calcoaceticus* (Ahmad *et al.*, 1988).

Western Blot Analysis

The $AroQ_p$-PheA samples were subjected to SDS-PAGE. After electrophoresis, the gels were blotted onto nitrocellulose membranes (Towbin *et al.*, 1979). The blots were then treated with the polyclonal antibody raised against the P-protein from *Acinetobacter calcoaceticus* (Ahmad *et al.*, 1988) followed by goat anti-(rabbit IgG) alkaline phosphatase conjugate. Nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) were used as chromogenic substrates for alkaline phosphatase.

Amino Acid Sequencing of the Cloned P-protein

The above IPTG-induced culture was harvested by centrifugation and resuspended in 50 m*M* potassium phosphate buffer (pH 7.0). The cells were broken by sonication, and 500 µl of the sample was transferred into a microcentrifuge tube, spun for 5 min at 200 x *g*. The pellet was resuspended in the same phosphate buffer. A 10-µl portion was mixed with 10 µl of SDS-PAGE buffer, boiled for 4 min, and subjected to SDS-PAGE. The protein was then transferred to a polyvinylidene difluoride membrane by a protein miniblotting apparatus and sequenced by using an Applied Biosystems model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyzer by the Protein Core Facility of ICBR at the University of Florida.

Molecular-mass Determination

The molecular mass of the native enzyme was determined by gel filtration using an FPLC-connected Superdex®75 HR 10/30 (Pharmacia Biotech) which had been previously equilibrated with 50 m*M* potassium phosphate buffer (pH 7.0) containing 1 m*M* tyrosine and 0.15 *M* NaCl; the same buffer was used for elution. The column was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,000), and alcohol dehydrogenase (150,000). V_o was determined with blue dextran (2,000,000). SDS-PAGE was carried out to determine the subunit molecular mass of the enzyme. Lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,000), and phosphorylase b (97,400) were used as molecular-mass standards.

Biochemicals and Chemicals

Ampicillin, gentamicin, thiamine, α-ketoglutarate, PMSF, amino acids and goat anti-(rabbit IgG) alkaline phosphatase were purchased from Sigma Chemical Company; NBT and BCIP were from Gibco-BRL; nitrocellulose membranes were from Micron Separations Inc.; DEAE-cellulose was from Whatman; hydroxylapatite was from Bio-Rad. Molecular-mass standards for gel filtration and for SDS-PAGE were from Sigma and Bio-Rad, respectively. Growth medium components and agar were from Difco. Prephenate and chorismate were prepared by this lab.

Nucleotide Sequence Accession Number

The nucleotide sequence reported in this communication has been assigned GenBank accession number U64518.

Results

Cloning of *aroQ*$_p$*-pheA* in *E. coli*

A random library generated via a partial digestion by *Sau*3A of *X. campestris* genomic DNA was constructed in *E. coli* in Dr. Gabriel's lab. Using the DNA from this library, six clones were obtained which carried plasmid inserts able to complement the *L*-phenylalanine auxotrophy of *E. coli* KA197 when cultured on minimal medium with gentamicin to select for maintenance of plasmid. The selection strategy could have yielded either *pheC* or *aroQ*$_p$*-pheA*. Expression of *pheC* would yield crude extracts showing prephenate dehydratase and arogenate dehydratase activities, whereas expression of *aroQ*$_p$*-pheA* would produce prephenate dehydratase activity only.

Clone pkao6, on these criteria, appeared to possess an *aroQ*$_p$-*pheA* insert. This was readily confirmed when anion-exchange chromatography (DEAE-cellulose) produced a fraction containing co-eluting chorismate mutase and prephenate dehydratase activities.

The pka06 clone contained a fragment of about 40 kb inserted in the *Bam*HI site of the pUFR043 cosmid. Upon digestion with *Eco*RI, fragments of 2.7 kb, 4.3 kb, >15 kb and >23 kb were obtained. When the 2.7-kb fragment was ligated into the *Eco*RI site of pUC18, it was successfully used for functional complementation of *E. coli* KA197. Enzymatic assays confirmed the presence of *aroQ*$_p$-*pheA* in strain KA197/pJG-XP1. Further subcloning experiments localized *pheA* within the *Pst*I-*Sac*II fragment of pJG-XP4 (Fig. 3-1). A comparison of the results obtained with pJG-XP2a and pJG-XP2b, which have opposite insert orientations, indicated either that a native promoter is absent in the insert or that the native promoter is not recognized by *E. coli*.

Sequence Analysis of *X. campestris aroQ*$_p$-*pheA*

Figure 3-2 shows the nucleotide sequence of *aroQ*$_p$-*pheA*, as well as some of the proximal flanking regions. The 1,200-bp open reading frame begins at codon ATG and terminates at codon TGA. The G+C content of *aroQ*$_p$-*pheA* (62.1%) is just

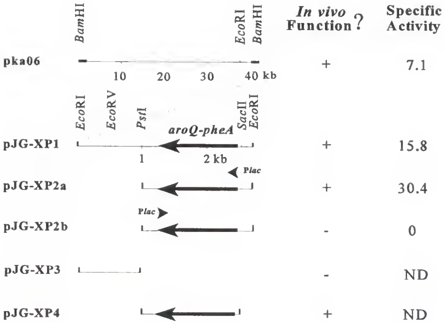


Fig. 3-1. Physical map and subclone analysis of *X. campestris* *aroQ*$_p$-*pheA*. The orientation of the *lac* promoter in the pUC18 vector is shown for the pJG-XP2a and pJG-XP2b subclones. Positive (+) *in vivo* function refers to the ability of a given clone to complement the *L*-phenylalanine auxotroph of *E. coli* KA197. Specific activities of prephenate dehydratase are given as nmole min^{-1} mg^{-1} at the right. ND, not done.

Fig. 3-2. Nucleotide sequence of *X. campestris* *aroQ*$_p$*-pheA* and its flanking regions. The probable ribosome binding site (rbs) is underlined. The 27-amino acid carboxy end of *serC* is shown at the top. Converging arrowheads mark a terminator structure between *serC* and *aroQ*$_p$*-pheA*. The first 10 amino acids of aroQ$_p$-pheA (bold) were confirmed by N-terminal amino acid sequencing. The regions corresponding to *aroQ*$_p$ and to *'pheA*, as marked, were deduced through multiple alignment analysis with monofunctional AroQ$_f$ and monofunctional PheA gene products. Nucleotide numbers assigned are shown at the far right.

```
-▸serC
GCGCTTCGCTGTACAACGCCATGCCGGTGGCCGGTGCGCAGGCACTGGTGGCCTTCATGCACGATTTCCAGCAGCGCCACGGCTGAGTCCCCCACAACCGCGGCATTGCCGCGCTGGCGC    120
 A  S  L  Y  N  A  M  P  V  A  G  A  Q  A  L  V  A  F  M  H  D  F  Q  Q  R  H  G  *                               >>>>>>
                               ┌▸aroQp domain
AGTGCGCCGTTTTATTGAAGGAAAGCCCCCGATGGCTCCCAAGCCCAAGAACACCAACGCCGCTGGCGGCACCGCCAAGTCGGCTACCAAGGCCAGCAGCAAGAAAGCCACCAAGTTGG     240
> <<<<<<<         rbs           M  A  P  K  P  K  N  T  N  A  A  G  G  T  A  K  S  A  T  K  A  S  S  K  K  A  T  K  L  A

CTGGCAAGGACAGCGCCAAGCCACTGGCAACCGCTGCCCCAGTGCTGGCCGATGTGCGTGCCAAGATCGACGAGATCGACCGTGGCATCCAGGCGTTGATCGCCGAGCGCGCCAACTTCG    360
 G  K  D  S  A  K  P  L  A  T  A  A  P  V  L  A  D  V  R  A  K  I  D  E  I  D  R  G  I  Q  A  L  I  A  E  R  A  N  F  A

CGCATCAGGTCGGCAAGGCCAAGGGCAAGCTGGCAGCGGCGGTGGATTACTACCGCCCCGAGCGCGAAGCGCAGGTGCTGCGCATGGTGGTGGACCGCAACGAAGGACCACTCAGCGATG    480
 H  Q  V  G  K  A  K  G  K  L  A  A  A  V  D  Y  Y  R  P  E  R  E  A  Q  V  L  R  M  V  V  D  R  N  E  G  P  L  S  D  E
                                                             ┌▸'pheA domain
AAGTGCTGGTGCATGTGTTCCGCGAAATCATGTCTGCGTGCCTTGCGCAGCAGGAGCCGCTGAAGATCGGGTATCTCGGCCCGGAAGGCACCTTCAGCCAGCAGGCGGTGCTCAAGCATT    600
 V  L  V  H  V  F  R  E  I  M  S  A  C  L  A  Q  Q  E  P  L  K  I  G  Y  L  G  P  E  G  T  F  S  Q  Q  A  V  L  K  H  F

TCGGCCGGTCGGCGGTGGGTCTGCCGATGGCGACCATCGAAGAAGTGTTTCAGGAAGTGGAAGCCGGCAATGCCGATTTCGGCGTGGTGCCGGTAGAAAATTCCGGCCAGGGCACGATCC    720
 G  R  S  A  V  G  L  P  M  A  T  I  E  E  V  F  Q  E  V  E  A  G  N  A  D  F  G  V  V  P  V  E  N  S  G  Q  G  T  I  Q

AGGTCACGCTGGACATGTTCCTCACCTCCAACCTCAAGATCTGCGGCGAAGTCGAGTTGCGTGTGCATCAGTACCTGCTTTCGCGCAACGGGCGCCTGGAAGACATCGAACGCATCTACG    840
 V  T  L  D  M  F  L  T  S  N  L  K  I  C  G  E  V  E  L  R  V  H  Q  Y  L  L  S  R  N  G  R  L  E  D  I  E  R  I  Y  A

CGCATTCGCAGTCGTTTGCGCAGACCGCCGGCTGGCTGCGTTCGCACTTGCCCAAGGTGGAAAAGATTGCGGTCTCCAGCAATGCCGAAGGCGCGCGCCGTGCGCGTAATGCCGAGGACG    960
 H  S  Q  S  F  A  Q  T  A  G  W  L  R  S  H  L  P  K  V  E  K  I  A  V  S  S  N  A  E  G  A  R  R  A  R  N  A  E  D  A

CGGCGGCCATCGGTGGCGAAAGCGCCGCGCACGTGTATGGCTTGAAAAAAGTCATCATGAAATCGATCGAGGACGACGACGACAACACCACGCGCTTTCTGGTGATCGGCCGGCAGATCT    1080
 A  A  I  G  G  E  S  A  A  H  V  Y  G  L  K  K  V  I  M  K  S  I  E  D  D  D  D  N  T  T  R  F  L  V  I  G  R  Q  I  F

TTCCATCGTCCGGGCATGACCGGACCTCGGTGCTGGTGTTCATCCACGACAAGCCGGGCGCGTTGTTCGATGTACTCAGCCCGTTCGCGCGGCATGGCATCAGCATGAACCGCATCGAGT    1200
 P  S  S  G  H  D  R  T  S  V  L  V  F  I  H  D  K  P  G  A  L  F  D  V  L  S  P  F  A  R  H  G  I  S  M  N  R  I  E  S

CGCGGCCGTCGCACCAGGCCAAGTGGGAATACGGCTTCTTTATCGACCTGGCCGGCCATGTGGAGGACGAGTCGATGAAACAGGCACTGGCCGAACTGGAAGCGCATTCGGCACAGATCA    1320
 R  P  S  H  Q  A  K  W  E  Y  G  F  F  I  D  L  A  G  H  V  E  D  E  S  M  K  Q  A  L  A  E  L  E  A  H  S  A  Q  I  K

AGGTGCTGGGCTCGTATCCGGTCGCCATTCCCTGAGCAGCGCATCGGCTGCGGCGCCGCGTCGGCCCTGACGCGGCGCCTGTCTCTACACCGCCTTCCTTCCCACGCGCATTGCTGACCG    1440
 V  L  G  S  Y  P  V  A  I  P  *

CAGCGATGTCGTCGCAATCGGATCATTCGCATGAGCAACAGCACGCAACACTGGATCGCACAGCGCGGCACCGCGCTGCAG                                           1548
```

outside the 63-72% range of *X. campestris*. The codon usage of *aroQ*$_p$*-pheA* is similar to that of rRNA-homology Group I pseudomonads (such as *P. aeruginosa*). The deduced amino acid sequence results in a 400-residue protein having a molecular mass of 43,428 Da. The highest pairwise identity is with the *P. stutzeri* homolog, a result consonant with the phylogenetic proximity of *X. campestris* and *P. stutzeri*.

A partial open reading frame encoding 27 residues was found upstream of *aroQ*$_p$*-pheA*. This gene is *serC* (encodes phosphoserine aminotransferase) in view of the high sequence identity (>50%) with the 3' termini from *serC* genes of both *Salmonella gallinarum* (Griffin and Griffin, 1991) and *Yersinia enterocolitica* (O'Gaora *et al.*, 1989). A factor-independent terminator structure is positioned at the immediate 5' side of the ribosome-binding site. Thus, there is no room for a promoter between the *serC* terminator and the ribosome-binding site.

Expression of *X. campestris aroQ*$_p$*-pheA* in *E. coli*

AroQ$_p$-PheA proved to be quite labile. Under various conditions where instability was encountered, both chorismate mutase and prephenate dehydratase activities were affected equally. Crude extracts containing AroQ$_p$-PheA were prepared from KA197/pJG-XP2. Preliminary purification led to complete loss of activity after the first step of several different fractionation protocols attempted. *L*-Tyrosine was known to be a stabilizing agent (Whitaker *et al*., 1985). Other conditions favoring stability were determined by testing additives which might protect AroQ$_p$-PheA activities in crude extract from heat inactivation. Crude extract in a basic 50 m*M* K-phosphate buffer at pH 7.0 was found to be subject to rapid inactivation at temperatures over 45°C. On the basis of protection conferred at a treatment temperature of 48°C, the components of the standard buffer (Methods) were adopted for routine use.

AroQ$_p$-PheA activities expressed by the *E. coli* clone were estimated to be 5-10 times greater than in the native organism. Sequential fractionation steps using $(NH_4)_2SO_4$ at 50% of saturation, DEAE-cellulose chromatography, and hydroxylapatite chromatography produced an additional overall purification of about 42-fold. SDS-PAGE analysis showed multiple bands. Any subsequent fractionation step, e.g., using Sephadex G-150, resulted in total loss of activity. Attempts to purify AroQ$_p$-PheA with protocols employing a different order of fractionation steps were no more successful. It therefore appeared that extreme lability resulted once a purification of about 40-fold was achieved.

In one experiment a partially purified preparation of AroQ$_p$-PheA from KA197/pJG-XP2 (after steps of $(NH_4)_2SO_4$

precipitation and DEAE-cellulose chromatography) was applied to a Sephadex G-150 column to estimate native molecular mass (data not shown). The active enzyme species is a homodimer, judging from the molecular mass determined (81,000 Da). In parallel gel-filtration runs where 1 m*M* *L*-tyrosine or 1 m*M* *L*-phenylalanine was present, 81,000-Da homodimers were again eluted.

Immunological Cross-reactivity

Antibody raised against *Acinetobacter calcoaceticus* AroQ_p-PheA had previously been found (Ahmad *et al.*, 1988) to cross-react strongly with homologs from *P. stutzeri* and *P. aeruginosa*, but only marginally with the homologs from several enteric bacteria. We found that anti-*A. calcoaceticus* AroQ_p-PheA antibody exhibited considerable cross-reactivity with *X. campestris* AroQ_p-PheA. Western-blot hybridization visualized a distinctive band at a M_r position of 43,000.

Overexpression of AroQ_p-PheA in *E. coli*

PCR was used to generate an *$aroQ_p$-pheA* insert for placement into the translation overexpression vector pET-11a. This construct (pJG-XP6) eliminated all native DNA sequence located upstream of the ATG start codon (Fig. 3-3A). pJG-XP6 was transformed into *E. coli* BL21(DE3), and a culture was supplemented with IPTG to induce the chromosomally encoded T_7

A

Overexpression vector (pJG-XP6):

T$_7$ promoter ↱*aroQ$_p$-pheA*
---▬----------GAAGGAGATATACAT**atggct---cctga**gcagcgcat---
S-D *NdeI*

---GGATCC---
BamHI

B

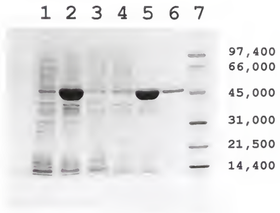


Fig. 3-3. Overexpression of AroQ$_p$-PheA from *X. campestris* in *E. coli*. A) Schematic representation of overexpression vector pJG-XP6. Uppercase letters represent the vector, lowercase letters represent the inserted *X. campestris* nucleotides, bold lowercase letters represent AroQ$_p$-PheA. The *Nde*I restriction site connects the vector and the 5' end of the AroQ$_p$-PheA insert, and the *Bam*HI restriction site is provided by the vector. B) SDS-PAGE analysis of AroQ$_p$-PheA expression in the following extracts. Lane 1, BL21(DE3)/pJG-XP6 (-IPTG); Lane 2, BL21(DE3)/pJG-XP6 (+IPTG); Lane 3, BL21(DE3)/pET11a (+IPTG); Lane 4, 200 x *g* supernatent from BL21(DE3)/pJG-XP6; Lane 5, 200 x *g* pellet from BL21(DE3)/pJG-XP6; Lane 6, 200 x *g* pellet from BL21(DE3)/pJG-XP6 at 10-fold dilution; Lane 7, Molecular-weight standards.

RNA polymerase. Chorismate mutase and prephenate dehydratase activities in crude extracts prepared from these cultures only increased over background about seven-fold. However, the pellet fraction obtained by the routine ultracentrifugation used to prepare crude extracts exhibited a prominent protein band at a M_r position of 43,000 Da following SDS-PAGE. The particulate $AroQ_p$-PheA could be collected by low-speed centrifugation (200 x *g*). When evaluated by SDS-PAGE, $AroQ_p$-PheA in the pellet fractions represented more than 90% of total protein (Fig. 3-3B). N-terminal amino acid sequencing of the excised band confirmed the identity of the protein as $AroQ_p$-PheA. The enzyme activity assayed in the particulate fractions was extremely high, more than 400-fold greater than that previously obtained with the KA197/pJG-XP2 construct. The enzyme activity assayed in the particulate fractions cannot be attributed to a contaminating aqueous component since passage through a Gelman Supor Acrodisc-13 syringe filter removed all enzyme activity.

None of $AroQ_p$-PheA preparations exhibited significant sensitivity inhibition by *L*-phenylalanine or to activation by *L*-tyrosine. Fig. 3-4 shows results of an experiment in which a substrate saturation curve for prephenate dehydratase was constructed in the presence of 5 m*M* *L*-phenylalanine. No inhibition was seen, even at low substrate concentration.

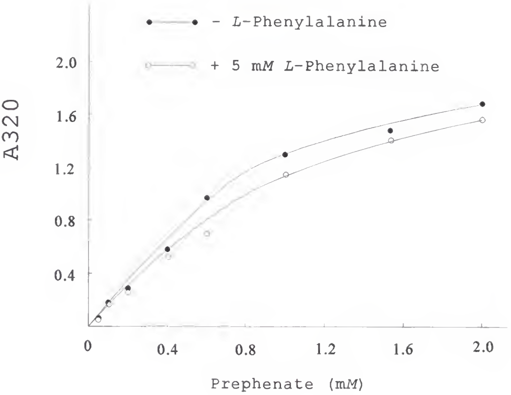


Fig. 3-4. Substrate saturation curve of prephenate dehydratase activity from *X. campestris* AroQ$_p$-PheA in the presence or absence of *L*-phenylalanine. Data shown were obtained from the particulate fraction recovered from BL21(DE3)/pJG-XP6.

Chorismate mutase activity was similarly invulnerable (data not shown). Soluble enzyme preparations from KA197/pJG-XP2 yielded similar results. Although *L*-tyrosine stabilizes AroQ_p-PheA, activation was not observed.

Visualization of Inclusion Bodies

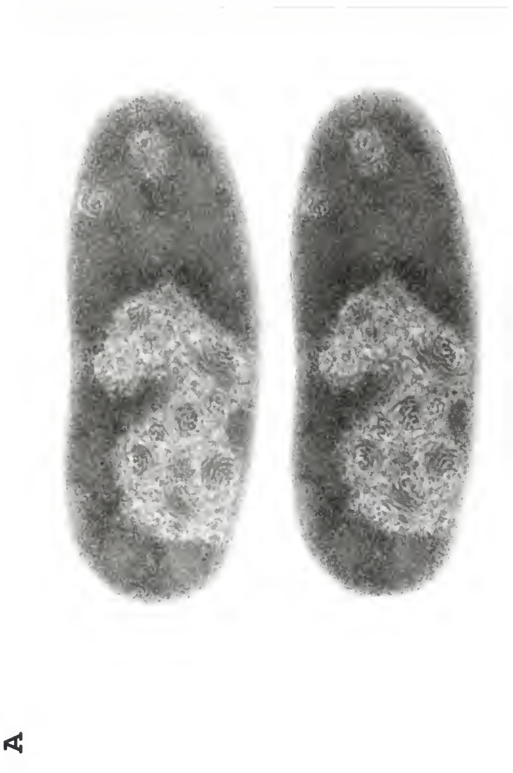
The insoluble character of AroQ_p-PheA suggested that it was probably sequestered in inclusion bodies, a supposition confirmed by electron microscopy (Fig. 3-5). The inclusion bodies were not round and refractile, often filled a substantial fraction of the cell volume, and were not membrane-bound. They exhibited a macromolecular structure manifesting spaghetti-like formations.

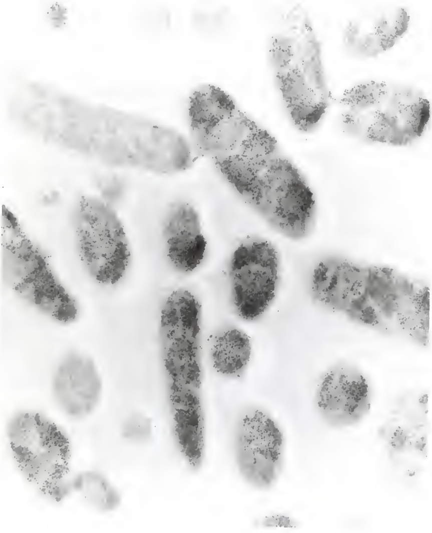
Discussion

Novel Features of AroQ_p-PheA

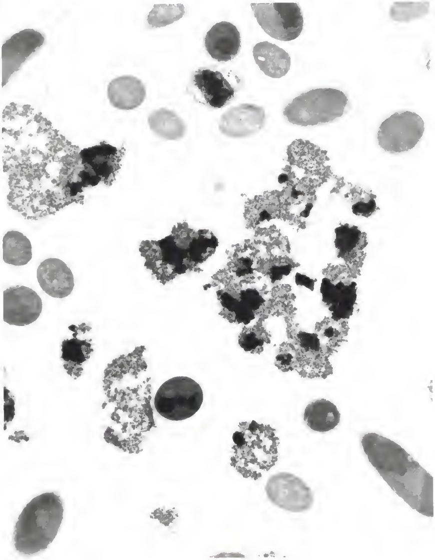
AroQ_p-PheA from *X. campestris* was obtained both as a soluble form at low expression levels and as a particulate form at high expression levels. SDS-PAGE of both forms indicated an identical subunit M_r. The soluble AroQ_p-PheA was found to be a homodimer in the presence or absence of *L*-tyrosine or *L*-phenylalanine. In *E. coli* and *E. herbicola* *L*-phenylalanine (but not *L*-tyrosine) promotes tetramerization of the native homodimer (Hudson and Davidson, 1984).

Fig. 3-5. Visualization of inclusion bodies by electron microscopy. A)negative stain of BL21(DE3)/pJG-XP6 whole cell showing spaghetti-like formations. B) immunogold labelling of inclusion body in BL21(DE3)/pJG-XP6 whole cell. C) negative stain of BL21(DE3)/pJG-XP6 crude extract showing released inclusion body. D) immunogold labelling of inclusion body released in BL21(DE3)/pJG-XP6 crude extract.


A

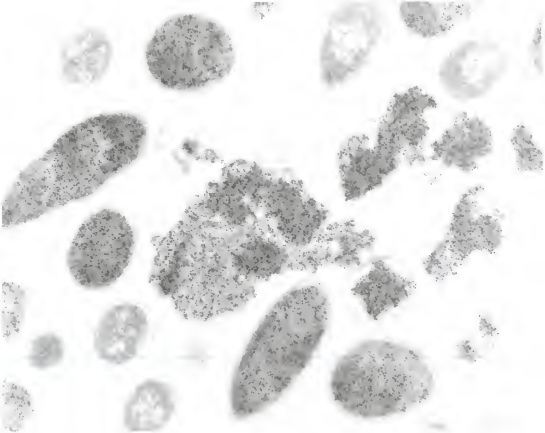


B



C

D



In *A. calcoaceticus* both *L*-tyrosine and *L*-phenylalanine promotetetramerization of a native homodimer (Ahmad *et al.*, 1988; and Berry *et al.*, 1985). In *Alcaligenes eutrophus* a native homotetramer was found to persist in the presence or absence of any effector molecule (Friedrich *et al.*, 1976). It is unfortunate that *A. calcoaceticus* and *A. eutrophus* *aroQ*$_p$-*pheA* genes have not yet been cloned, in part because of their appropriate phylogenetic distance and in part because they have been thoroughly characterized at the enzymological level. The molecular-genetic underpinnings of the considerable diversity of physical and catalytic properties of AroQ$_p$-PheA proteins throughout the ß and γ divisions of gram-negative bacteria will be of great interest.

The 40-amino acid extension at the amino terminus of *X. campestris* AroQ$_p$-PheA is thus far unique among the homologs currently available. Its high lysine content confers a potent positive charge at the amino terminus. Water molecules retained by this hydrophilic region might provide an aqueous layer that could account for the enzyme activity of the insoluble aggregate of *X. campestris* AroQ$_p$-PheA. The positively charged terminus could also act as a funnel to attract chorismate molecules (with their two negatively charged carboxyl moieties) to the specific microenvironment of the AroQ$_p$ catalytic site. It is possible that the extension has some unknown function that is unique to the native organism. It might be processed by proteolytic cleavage to a mature species not expressed in *E. coli*.

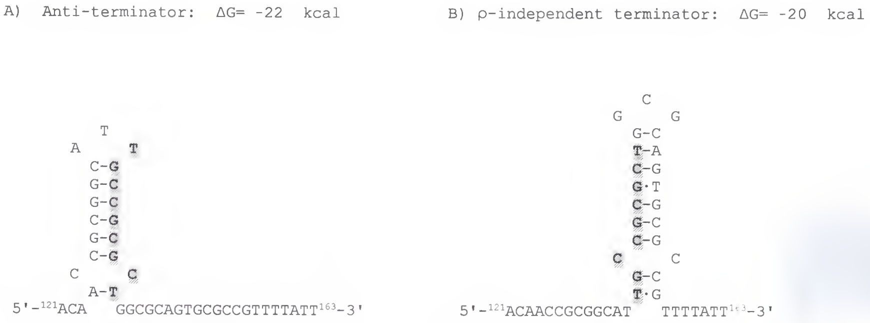
The various AroQ$_p$-PheA proteins differ in ability to interact with *L*-tyrosine. At one extreme, e.g., in enteric bacteria (Hudson and Davisdon, 1984), there is no detectable effect of *L*-tyrosine as an allosteric agent or protectant. At the other extreme, AroQ$_p$-PheA is totally inactive in the absence of *L*-tyrosine, e.g., *Alcaligenes faecalis* and *Nitrosomonas europaea* (Subramaniam *et al.*, 1994). Throughout the ß and γ subdivisions of the Proteobacteria, other AroQ$_p$-PheA proteins exhibit a wide range of sensitivity to *L*-tyrosine as an activator. In organisms such as *Neisseria gonorrhoeae* and *Pseudomonas saccharophila*, dramatic activation on the order of 20-fold is observed (Subramaniam *et al.*, 1994). In contrast, organisms such as *Rhodocyclus tenuis* (Subramaniam *et al.*, 1994) resemble *X. campestris* in showing only modest activation effects of 10% or less. In *X. campestris* interaction with *L*-tyrosine is most apparent from its efficiency as a stabilizing agent.

Gene Organization and Regulation

In enteric bacteria (*E. coli* and *E. herbicola*) AroQ$_p$-PheA is regulated by attenuation, which depends upon a *L*-

phenylalanine-rich leader peptide encoded by *pheL* (Hudson and Davidson, 1984; and Xia *et al.*, 1993a). *X. campestris* lacks such an upstream gene encoding a leader peptide. However, the limited intercistronic space indicates that the promoter for *aroQ*$_p$-*pheA* must be ahead of or within the upstream gene, *serC*. Alternative stem-loop structures exist between the two genes, as shown in Fig. 3-6, which may reflect a mechanism of regulation by attenuation. The *serC-pheA* gene organization is also maintained in the relatively close phylogenetic neighbor, *P. stutzeri*. In the latter case there is no intercistronic space, and *serC* and *aroQ*$_p$-*pheA* are translationally coupled (Fischer *et al.*, 1991). Again, alternative stem-loop structures exist, one of which sequesters the *aroQ*$_p$-*pheA* ribosome binding site. Thus, yet another form of attenuation may be used.

In enteric bacteria, *serC* and *aroE* are organized as a mixed-function operon and transcribed from a common promoter (Duncan and Coggins, 1986; Griffin and Griffin, 1991; and O'Gaora *et al.*, 1989). In *H. influenzae serC* may be organized (Fleischmann *et al.*, 1996) with *hisH*, a broad-specificity aminotransferase that can transaminate aromatic amino acids (Jensen and Gu, 1996). Thus, for unknown reasons, *serC* appears to be commonly organized within mixed-function operons in which the second gene participates in aromatic amino acid



A) Anti-terminator: ΔG= -22 kcal

B) ρ-independent terminator: ΔG= -20 kcal

T
A T
C-G
G-C
G-C
C-G
G-C
C-G
C C
A-T
5'-121ACA GGCGCAGTGCGCCGTTTTATT163-3'
C
G G
G-C
T-A
C-G
G·T
C-G
G-C
C-G
C C
G-C
T·G
5'-121ACAACCGCGGCAT TTTTATT163-3'

Fig. 3-6. Alternative stem-loop structures between *serC* and *aroQ*$_p$-*pheA*. The shaded base residues are common to both the factor-independent terminator structure shown in **B**), and to the anti-terminator structure shown in **A**).

biosynthesis. It is interesting that in *P. stutzeri* the gene immediately downstream of *serC/aroQ*$_p$*-pheA* is *hisH* (Xie and Jensen, unpublished data).

The *aroQ* Gene Family

Chorismate mutase is a facile reaction which appears to have evolved independently at least three times. X-ray crystallographic analysis has revealed structurally different catalytic centers for *E. coli* AroQ$_p$ (Lee *et al.*, 1995), and *B. subtilis* AroQ$_f$ (encoded by *aroH*) (Chook *et al.*, 1993). Yeast AroQ$_r$ possesses a catalytic center that is structurally similar to that of *E. coli* AroQ$_p$, but the primary sequences do not exhibit homology (Sträter *et al.*, 1996). *B. subtilis* AroQ$_f$ has no known homologs, and the AroQ$_r$ family consists of two homologs, one from *Saccharomyces* and one from *Arabidopsis thaliana*. The current AroQ gene family has eleven members, as shown by the multiple alignment in Fig. 3-7. Nine members of the family display a catalytic domain for chorismate mutase that coexists on the same protein with another catalytic domain. The second catalytic domain of these bifunctional proteins is either prephenate dehydratase (five sequences), prephenate dehydrogenase (three sequences), or 3-deoxy-*D*-*arabino*-heptulosonate 7-P synthase (one sequence). A monofunctional AroQ$_f$ from *Methanococcus jannaschii*, an

FIG. 3-7. Multiple alignment of the AroQ protein family. The most divergent member of the family, Ehe AroQ$_f$, is spaced at the bottom from other family members. Residues located at the active site of Eco AroQ$_p$, as demonstrated by X-ray crystallography (bottom schematic, from Lee *et al.*, 1995), are indicated by residue numbers at the top. Residue 11' is located on the other subunit, with respect to other residues, in the active homodimer. Residues conserved throughout at least the major cluster are boxed. Residues conserved in all but one sequence are shaded. Residues marked with an H at the top participate in important hydrophobic interactions is Eco AroQ$_p$. Residue numbers are shown at the far right. Abbreviations: Pst, *P. stutzeri*; Xca, *X. campestris*; Bsu, *Bacillus subitilis*; Eco, *E. coli*; EHe, *Erwinia herbicola*; Hin, *Haemophilus influenzae*; and Mja, *Methanococcus jannaschii*

```
                            11'                                   28              H       39
                            ▲                                     ▲               ▲       ▲
Pst AroQ_p  E A D Q L K A L R V   R I D S L D E R I L   D L I S E R A R C A   Q E V A R V K T A S   W P K A E E A V F Y   52
Xca AroQ_p  A A P V L A D V R A   K I D E I D R G I Q   A L I A E R A N F A   N Q V G K A K G . .   . K L A A A V D Y Y   87
Bsu AroQ_d  S N T E L E L L R Q   K A D E L N L Q I L   K L I N E R G N V V   K E I G K A K E .     . . . A Q G V N R F   46
Eco AroQ_t  M V A E L T A L R D   Q I D E V D K A L L   N L L A K R L E L V   A E V G E V K S R F   . . . . . G L P I Y   45
Ehe AroQ_t  M V A E L T A L R D   Q I D S V D K A L L   D L L A K R L E L V   A E V G E V K S R Y   . . . . . G L P I Y   45
Hin AroQ_t  F M E A L K D L R S   E I D S L D R E L I   Q L F A K R L E L V   S Q V G K V K H Q H   . . . . . G L P I Y   47
Eco AroQ_p  S E N P L L A L R E   K I S A L D E K L L   A L L A E R R E L A   V E V G K A K L L S   . . . . . N R P V R   47
Ehe AroQ_p  P D N P L L A L R D   K I S A V D K K L L   T L L A E R R L L A   V E V A Q A K L A T   . . . . . N R P I R   47
Hin AroQ_p  M A L E L S D I R Q   Q I T Q I D R S L L   K L L S E R N R L A   F D V V R S K E I S   . . . . . Q K S L R   45
Mjs AroQ_f  M I E K L A E I R K   K I D E I D N K I L   K L I A E R N S L A   K D V A E I K N Q L   . . . . . G I P I N   45

Ehe AroQ_f  M C S N V F A G S V   S S V S L G . S L S   S A L N E R M Q V M   K A V A G Y K A L .   . . . . N N L P I E   57

            48   51 52    H                                                             H    84 H       88
            ▲     ▲ ▲     ▲                                                             ▲     ▲ ▲       ▲
Pst AroQ_p  R P E R E A W V L K   H I M E L N K G . .     P L D N E E M A R   L F R E I M S S C L   A L E Q P L .         95
Xca AroQ_p  R P E R E A Q V L R   M V V D R N E G . .     P L S D E V L V N   V F R E I M S A C L   A Q Q E P L .         130
Bsu AroQ_d  D P V R E R T M L N   N I I E N N D G . .   . P F E N S T I Q N   I F K E I F K A G L   E L Q E E D H         90
Eco AroQ_t  V P E R E A S M L A   S R R A . . E A E A   L G V P P D L I E D   V L R R V M R E S Y   S S E N D K G         90
Ehe AroQ_t  V P E R E A S M L A   S R R K . . E A E A   L G V P P D L I E D   V L R R V M R E S Y   T S E N D K G         90
Hin AroQ_t  A P E R E I A M L Q   A R R L . . E A E K   A G I S A D L I E D   V L R R F M R E S Y   A N E N Q F G         92
Eco AroQ_p  D I D R E R D L L E   R L I T L G K A N N       L D A H Y I T R   L F Q L I I E D S V   L T Q Q A L L         92
Ehe AroQ_p  D V E R E R A L L E   N L I V L G K A H N   . . L D A H Y I T R   L F Q L V I E D S V   L T Q Q A L L         92
Hin AroQ_p  D V E R E Q Q L L Q   E L V Q F A E N E N   Y Q L E A Q Y I T S   I F Q K I I E D S V   L T Q Q V Y L         92
Mjs AroQ_f  D P E R E K Y I Y D   R I R K L C K E H N   .   V D E N I G I K   I F Q I L I E H N K   A L Q K Q Y L         90

Ehe AroQ_f  D L P R E Q V V L D   N M L Q . . N A Q Q   A G L E P H S V E P   F V N A L M N A S K   T I Q Y R Y R         102
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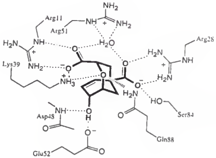

archaeon representative, is remarkably conserved (Bult *et al.*, 1996) with respect to important catalytic residues. $\mathrm{AroQ_f}$ from *E. herbicola*, the second monofunctional species, exhibits the least overall identity with the other family members (including two paralog sequences from *E. herbicola*). This may reflect a divergence related to the periplasmic location (Xia *et al.*, 1993b) of this species. Nevertheless, *E. herbicola* $\mathrm{AroQ_f}$ exhibits conservation of all but one of the important catalytic residues established for *E. coli* $\mathrm{AroQ_p}$.

Figure 3-7 includes a schematic diagram illustrating the hydrogen bonding and electrostatic interactions of a transition state analog (*endo*-oxabicyclic inhibitor) with sidechains of eight catalytic-site residues of *E. coli* $\mathrm{AroQ_p}$ (Lee *et al.*, 1995). Of these, Arg51, Arg28, Glu52 and Lys39 are conserved throughout the family. Only Ehe-$\mathrm{AroQ_f}$ lacks Arg11' which contacts the oxygen atoms of the carboxyl moiety of the enolpyruvyl sidechain. Ser84 and Asp48 are not highly conserved. In those cases where position 88 is not occupied by Gln, it is Glu88. Interestingly, when Eco-$\mathrm{AroQ_p}$ Gln88 was replaced by Glu88, 1% of normal catalytic activity was retained at pH 7.8. However, at pH 4.5 the Q88E mutant exhibited 140% of wildtype activity (Zhang *et al.*, 1996). Low pH presumably allows the undissociated carboxylic acid of Glu88 to function as a hydrogen bond donor, in concert with

Lys39 (Zhang *et al.*, 1996). Further affirmation of this is that yeast chorismate mutase, which has a glutamate residue in the equivalent position (Sträter *et al.*, 1996), exhibits a pH optimum of 5.5 with little activity at pH 7.8. Thus, one might expect the E88 and Q88 members of the family to exhibit low-pH and high-pH optima, respectively.

The *pheA* Gene Family

Prephenate dehydratase is represented in nature either as a monofunctional protein (PheA) or as one catalytic domain of bifunctional proteins (AroQ$_p$-PheA). A multiple alignment is shown in Fig. 3-8 of 10 sequences, five of them monofunctional proteins (PheA) and five of them PheA domains (ʻPheA). All ʻPheA domains are from the γ division of gram-negative bacteria. ʻPheA sequences from representatives of the ß division are not yet available (although AroQ$_p$-PheA proteins from members of this division have been well studied (Friedrich *et al.*, 1976; and Subramaniam *et al.*, 1994). Sequences for the monofunctional PheA (and AroQ) proteins present in the α division are also not yet represented. All previously reported dehydratase proteins are feedback inhibited by *L*-phenylalanine. The carboxy end of prephenate dehydratase proteins is a discrete location for feedback inhibition, based upon deletion and mutant analysis in *aroQ*$_p$-

FIG. 3-8. Multiple alignment of monofunctional prephenate dehydratases (PheA) and P-protein prephenate dehydratase domains ('PheA). Completely conserved residues are boxed, and highly conserved residues are shaded. The junction separating the catalytic and allosteric regions are indicated. Residues 309 and 338, shown in *E. coli* to be important for feedback inhibition are marked. A region suggested to be responsible for tyrosine-mediated activation is double-underlined and marked with a circled 'plus'.

```
Cgl  PheA   VVAYLGPAGT  FTEEALYKFA  DAGVFGDGEI  EQLPAKSPQE  AVDAVRHGTA  QFAVVAIENF   66
Ame  PheA   RIAYFGPVGT  FTEQAARTFM  AAG .. ..D  ELVAAETIPK  ALDAVRRE.A  DAACVPVENS   55
Eco  PheA   RIAFLGPKGS  YSHLAARQYA  A.. RHFEQF  IESGCAKFAD  IFNQVETGQA  DYAVVPIENT  161
Ehe  PheA   RIAFLGPKGS  YSHLAARNYA  S  .RHFDSM  VECGCLKFHD  IIKQVENGVA  DYAVMPIENT  161
Hin  PheA   HIAFLGKRGS  YSNLAARNYA  A   RYQKQF  VELGCQSFEQ  VFEKVQTGEA  DPGVLPLENT  162
Pst  PheA   RVAYLGPEGT  FSQAAALKHF  G   H . SV  ISKPMAAIDE  VFREVVAGAV  NFGVVPVENS  149
Xca  PheA   KIGYLGPEGT  FSQQAVLKHF  G   R   SA  VGLPMATIEE  VFQEVEAGNA  DFGVVPVENS  184
Bsu  PheA   KVGYLGPAAT  FTHLAVSSCF  Q   NGAEHV  A.. YRTIPE  CIDAAVAGEV  DFAFVPLENA   55
Lla  PheA   KIAYLGPRGS  FCSVVAEAAF  .   KSEELY  S. .YATILD  VIEAYNEGEC  DFALVPIENS   54
Mja  PheA   AVIYTLPKGT  YSEKATKKFL  D   YIDGDY  KIDYCNSIYD  VFE  RVDNN  GLGVVPIENS   58

Cgl  PheA   VDGPVTPTFD  ALDQGSNVQI  IAEEELDIAF  SISV  RPGT  SLADVKTLAT  HPVGYQQVKN  124
Ame  PheA   VEGAVPATLD  SLAVGEPLIG  VAEALLPVHF  SVLT  RDD    VGEIRTVAS  HPHALAQVRK  111
Eco  PheA   SSGAINDVYD   LLQHTSLSI  VGEMTLTIDH  CLLV  SGTT  DLSTINTVYS  HPQPFQQCSK  218
Ehe  PheA   SSGSINDVYD   LLQQTSLSI  VGELTLPIDH  CVLV  NGPT  DLQQIETVYS  HPQPFQQCSQ  218
Hin  PheA   TSGAINEVYD   LLQHTDLSL  VGELAYPIKH  CVLV  NDKT  DLNQIDTLYS  HPQVIQQCSQ  219
Pst  PheA   TEGAVNHTLD   SFLEHDIVI  CGEVELRIHH  HLLV  GETT  KTDRITRIYS  HAQSLAQCRK  206
Xca  PheA   GQGTIQVTLD  .MFLTSNLKI  CGEVELRVHQ  YLL.  SRNG  RLEDIERIYA  HSQSFAQTAG  239
Bsu  PheA   LEGSVNLTID  YLIHEQPLPI  VGEMTLPIHQ  HLLVHPSREN  AWKELDKIYS  HSHAIAQCHK  115
Lla  PheA   TEGTVNMSID  KIFHDSNAKV  VAEFVLPISQ  NLLA VSKE    .QKIEHIYS  HPQALAQTRV  110
Mja  PheA   IEGSVSLTQD  LLLQFKDIKI  LGELALDIHH  NLIGY       DKNKIKTVIS  HPQALAQCRN  113

Cgl  PheA   WMATTIPDAM  YLSASSNGAG  AQMVAE  GT  ADAAAAPSRA  AELFGLERLV  D DVADVRGA  181
Ame  PheA   WLEDNLPGAR  VVAAGSTAAA  AVAVQA  GE  FDAAVTAPVA  VEHYPLKVLA  T EVADVRDA  168
Eco  PheA   FLNRYPH WK  IEYTESTSAA  MEKVAQAKSP  HVAALGSEAG  GTLYGLQ VL  ERIEANQRQN  276
Ehe  PheA   FINRFPH WK  IEYTESTAAA  MEKVAALNSP  KVAALGSEAG  GELYQLQ VL  ERNLANQQQN  276
Hin  PheA   FIHSLDR VH  IEYCESSSHA  MQLVASLNKP  NIAALGNEDG  GKLYGLS VL  KTNIANQENN  277
Pst  PheA   WLDAHYP  N  VERVAVSSNA  DAAKRVKSEW  NSAAIAGDMA  AQLYGLSKLA  EK IEDRPDN  263
Xca  PheA   WLRSHLP  K  VEKIAVSSNA  EGARRARNAE  DAAAIGGESA  AHVYGLKKVI  MKSIEDDDDN  297
Bsu  PheA   FLHRHFPSVP  YEYANSTGAA  AKFVSDHPEL  NIGVIANDMA  ASTYELK IV  KRDIQDYRDN  174
Lla  PheA   YLRKFYPQAQ  VEITESTSAA  AEFVKNNPDL  PAAAVANSFA  AKMYDLE FI  AENIQDLAGN  169
Mja  PheA   YIKKH  GWD  VKAVESTAKA  VKIVAESKDE  TLGAIGSKES  AEHYNLK IL  DENIEDYKNN  170

       Catalytic domain ◄─┐               ┌─► Allosteric domain
Cgl  PheA   RTRFVAVQAQ  A      AV   SEPTGHDRTS  VIFSLPNVPG  SLVRALNEFG  IRGVDLTRIE  234
Ame  PheA   RTRFLLMRRP  P      VVL  PEPTGADRTS  IVAAAANRTG  TLAELLTELA  TRGINLTRLD  222
Eco  PheA   FTRFVVLARK  A       IN  VSDQVPAKTT  LLMATGQQAG  ALVEALLVLR  NHNLIMTRLE  329
Ehe  PheA   HTRFIVLARK  A       IE  VSDQVPAKTT  LIMATGQQAG  ALVDALLVLR  QHNLIMSKLE  329
Hin  PheA   ITRFIVVAKE  P       RE  VSSQIPTKTL  LLMTTSQQAG  ALVDALLVFK  KHQINMTKLE  330
Pst  PheA   STRFLIIGSQ  E              VPPTGDDKTS  IIVSMRNKPG  ALHELLMPFH  SNGIDLTRIE  314
Xca  PheA   TTRFLVIGRQ  I       .   FPSSGHDRTS  VLVFIHDKPG  ALFDVLSPFA  RHGISMNRIE  348
Bsu  PheA   HTRFVILSPD  ENISFEVNSK  LSSRPKTTLM  VMLPQDDQSG  ALHRVLSAFS  WRNLNLSKIE  234
Lla  PheA   STRFWLLG K  EKQSFDLN     QTKDKVTLA  LTLP.DNLPG  ALHKAISVFA  WRDIDMTKIE  224
Mja  PheA   KTRFILIGKK  VKFKYH        PKNYKVSIV  FEL.KEDKPG  ALYHILKEFA  ERNINLTRIE  224
                                       ════
                                        ⊕               ▼
                                                       309

Cgl  PheA   SRPTRKVFGT  YRFHLDISGH  .IRDIPVAEA  LRALHLQAEE  LVFVGSWPSN  283
Ame  PheA   ARPHKQNFGE  YRFFIDFEGH  .VAEPRIADA  LAALRRRCRD  VRFLGSFA.   269
Eco  PheA   SRPIHGNPWE  EMFYLDIQAN  .LESAEMQKA  LKELGEITRS  MKVLGCYPSE  378
Ehe  PheA   SRPINGNPWE  EMFYIDVQGN   LQSERMQQA  LQELQTMTRS  LKVLGCYPSE  378
Hin  PheA   SRPIYGKPWE  EMFYLEIEAN  .IHHPDTKQA  LEELKNYSNY  LKILGCYPSE  379
Pst  PheA   TRPSRSGKWT  YVFFIDCMGH  .YQDPLIKDV  LEKIDHEAVA  LKVLGSYPKA  363
Xca  PheA   SRPSHQAKWE  YGFFIDLAGH   VEDESMKQA  LAELEAHSAQ  IKVLGSYPVA  397
Bsu  PheA   SRPTKTGLGH  YFFIIDIE K  AFDDVLIPGA  MQELEALGCK  VRLLGAYQSY  283
Lla  PheA   SRPLRTRLGQ  YFFIIDLENN  ATNSLKIPYA  LEELAGLGVN  VRLLGNYSVY  274
Mja  PheA    RPSKKRLGT  YIFYIDFEN     NKEKLEEI  LKSLERHTTF  INLLGKYPVF  271
                    ▼
                   338
```

pheA from *E. herbicola* (Xia *et al.*, 1992) and *E. coli* (Nelms *et al.*, 1992). Fig. 3-8 shows an approximate demarkation between the N-terminal catalytic domain and the C-terminal allosteric domain based upon the location of known mutations which affect catalysis or allostery independently of one another. The conservation of various residues throughout the homology family indicates that all share the N-catalytic/C-allostery organization. It has been suggested (Vrijbloed *et al.*, 1995) that the motif PTGxD marked in Fig. 3-8 might correspond to allosteric activation by tyrosine found in Cgl PheA (Follettie and Sinskey, 1986), Ame PheA (Vrijbloed *et al.*, 1995) and *P. stutzeri* PheA (Byng *et al.*, 1983). The minimal activation, but stablilization, conferred by *L*-tyrosine in *X. campestris* (Whitaker *et al.*, 1985) is consistent with the different but similar motif **SSGxD** which might allow *L*-tyrosine binding, but not activation.

Bacillus subtilis PheA is known to exhibit additional allosteric effects which include inhibition by *L*-tryptophan and activation by *L*-methionine and *L*-leucine. Activator and inhibitor molecules induce transitions of molecular mass, to dimers and octamers respectively (Pierson and Jensen, 1974). The molecular basis of this complexity in relationship to homologs which are only affected by *L*-phenylalanine would be of interest.

GLOSSARY

P-protein	Bifunctional protein containing a chorismate mutase domain and a prephenate dehydratase domain.
T-protein	Bifunctional protein containing a chorismate mutase domain and a prephenate dehydrogenase domain.
pheA	Encodes monofunctional prephenate dehydratase.
'pheA	Encodes the prephenate dehydratase domain of a P-protein.
tyrA	Encodes monofunctional prephenate dehydrogenase.
'tyrA	Encodes the prephenate dehydrogenase domain of a T-protein.
$aroQ_f$	Encodes the monofunctional, allosterically-insensitive type of chorismate mutase.
$aroQ_r$	Encodes the monofunctional, allosterically-sensitive type of chorismate mutase.
$aroQ_p$	Encodes the chorismate mutase domain of bifunctional P-protein.
$aroQ_t$	Encodes the chorismate mutase domain of bifunctional T-protein.
$aroQ_d$	Encodes the chorismate mutase domain coexisting with a DAHP synthase domain on a bifunctional protein.
pheC	Encodes cyclohexadienyl dehydratase.
tyrB	Encodes aromatic amino acid aminotransferase in *E. coli*.
aspC	Encodes aspartate aminotransferase in *E. coli*.
ilvE	Encodes branched-chain amino acid aminotransferase in *E. coli*.
phhC	Encodes an aromatic aminotransferase in *P. aeruginosa*.

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BIOGRAPHICAL SKETCH

Wei Gu was born on March 20, 1964, in Shanghai, China. She completed her elementary and high school education in Jinan, Shandong Province. In 1981, she enrolled in the Shandong University, where she majored in microbiology. In 1985, she received her B.S. degree. In the same year, she attended the Shanghai Institute of Plant Physiology, Academia Sinica, and worked there until 1988. In 1989, she joined the research group of Dr. Roy A. Jensen where she pursued enzymological and molecular genetic studies of the imidazole acetol phosphate aminotransferase in *Zymomonas mobilis*. Under the supervision of Dr. Roy A. Jensen, she received her M.S. degree in August, 1993. She then continued with her Ph.D. program of studies, focused on aspects of phenylalanine biosynthesis in *Pseudomonas aeruginosa* and *Xanthomonas campestris,* receiving her Ph.D. degree in May, 1997. She started her postdoctoral research at Rice University in January, 1997.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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